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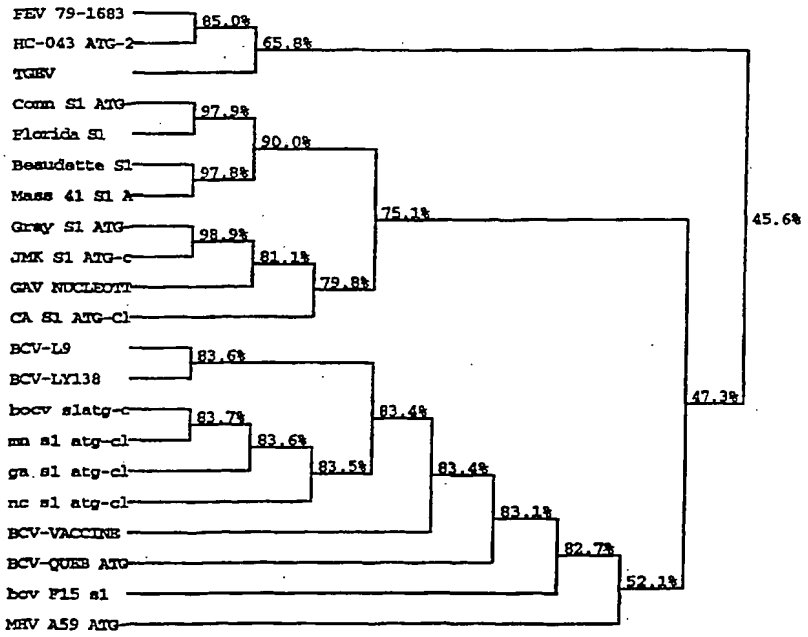

(57) Abstract

A preselected DNA segment encoding turkey enteric coronavirus S1 polypeptide is provided. Also provided are methods to inhibit or prevent spiking mortality of turkeys (SMT). Further provided are immunogenic compositions comprising cell culture adapted coronaviruses. A method to detect the presence of turkey enteric coronavirus or antibodies thereto in a sample derived from a bird or mammal is also provided.

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COMPOSITIONS AND METHODS TO PREVENT TURKEY ENTERITIS

Background of the Invention

This application claims the benefit of U.S. Provisional Application Serial Number 60/065,556 filed November 14, 1997 under 35 USC119(e). In the fall of 1991, a previously unrecognized syndrome characterized by acute enteritis, bursal atrophy, and thymic atrophy was first identified in multiple commercial turkey houses. Affected growers termed the syndrome Spiking Mortality of Turkeys (SMT) (Brown et al., 1992). Coronavirus, alphavirus, cryptosporidiosis, cochlasoma, hexamita, and reovirus have been considered as possible causes for SMT (Ficken et al., 1993), and other viral agents have been documented to produce milder enteritis in young turkey poults (Reynolds et al., 1987).

After further study, the defining features of SMT were found to be: the rapid onset of mortality of over 1% of the flock per day for 1 to 5 days in commercial turkey poults 5 to 25 days old; acute severe intestinal villus atrophy and crypt hyperplasia; bursal atrophy with production of bursal cores of inspissated pus; severe thymic atrophy; cell-mediated immunosuppression; failure of survivors to gain weight normally; hot weather seasonality; and frequent reoccurrence on affected farms (Brown et al., 1993).

SMT has recently been reclassified as the most severe form of the more inclusive Poult Enteritis and Mortality Syndrome (PEMS). Poult Enteritis and Mortality Syndrome/Spiking Mortality of Turkeys (PEMS/SMT) has emerged as the largest threat to the turkey industry in the last decade. Severe outbreaks are characterized by 30% to 70% mortality, but milder forms associated with stunting and poor feed conversions have had equally severe economic consequences.

PEMS/SMT is infectious and transmissible, but the specific cause(s) remains unidentified. It is spread by contact with litter or feces and is carried by recovered turkeys. Diligent decontamination efforts and biosecurity have failed

to eliminate or to limit the slow spread of PEMS/SMT from contaminated premises.

Turkey enteric coronaviruses are one of the major causative agents of diarrhea in turkey poults (Dea et al., 1985; Dea et al., 1990b). In the early 1970s, a coronavirus was identified as the primary etiologic agent of Bluecomb Disease or Transmissible Enteritis of Turkeys (Naqi et al., 1975; Pensaert et al., 1994; Pomeroy, 1980). Although there are similarities between PEMS/SMT and Transmissible Enteritis of turkeys, remarkable differences are evident, namely, the time of the year when the outbreaks are experienced and the severe damage to the bursa and thymus of the affected birds with PEMS/SMT (Brown et al., 1993). The failure to eliminate or inhibit the spread of PEMS/SMT suggests that subclinical carriers exist, and that these carriers, along with insects and possibly other animal species, may serve as sites of subsequent spread to other farms, and recontamination of affected farms.

Thus, what is needed is the identification and isolation of the infectious agent associated with SMT. Moreover, there is a need for a method to detect the presence of the infectious agent in animals, as well as a need for immunogenic compositions useful to prevent or inhibit SMT.

Summary of the Invention

In order to identify and characterize the infectious agent which causes or is associated with SMT, infected material, e.g., feces or organ homogenates, was used to infect poults. Infected material was also clarified and passaged through cell culture. A coronavirus, turkey enteritis coronavirus (TCV), was identified as an infectious agent that was associated with SMT. Four such isolates were propagated in monolayers of a continuous human rectal tumor cell line (HRT-18). Antibody to the reference Minnesota strain of turkey coronavirus (TCV-MN) cross-reacted with the new turkey enteric isolates. Unlike previously identified TCV isolates (e.g., TCV-MN), the TCV isolates described herein can routinely be propagated in cell culture for greater than 1-4 passages.

Once a correlation has been established between the presence of particular isolates of TCV and SMT, physiological samples, e.g., tissues, blood

sera or plasma, from an afflicted or exposed turkey or an organism suspected of being a carrier of TCV, can be analyzed for the presence of coronavirus, viral-specific nucleic acid, e.g., by an amplification reaction such as the polymerase chain reaction, antibodies specific for a virally encoded polypeptide or antibodies specific for the coronavirus. The presence or amount of the virus, genomic viral nucleic acid, viral polypeptide or viral-specific antibodies in the sample can then be compared to a control sample, e.g., from a disease-free organism.

Thus, the present invention provides isolated preparations or compositions comprising a cell culture adapted TCV isolate obtained from a turkey having SMT, or a multicellular organism exposed to a turkey having SMT. The preparations or compositions of the invention are substantially free from other infectious agents. As used herein, "substantially free" means below the level of detection for a particular infectious agent using standard detection methods for that agent. Preferably, the cell cultures infected with these TCV isolates produce titers of virus useful to prepare killed coronaviral vaccines or derive modified-live (attenuated) vaccines, to prepare antibodies useful for disease prevention (passive immunization), and for diagnostics. For example, killed virus can be administered to hens for vertical transmission. Progeny with maternal antibody can then be vaccinated with live virus for active immunity. Preferred TCV isolates include TCV-NC (also termed UGA-APN herein), TCV-GA (also referred to as UGA-APG herein), TCV-BOB (also termed UGA-APBOB), and TCV-BOA (also referred to as UGA-APBOA).

Classical Minnesota-type isolates of turkey intestinal coronaviruses (TCV-MN) follow the pattern of other enteropathogenic coronaviruses and produce jejunal villus atrophy, dehydration and death in neonates. Recovered individuals are refractory to re-infection due to mucosal immunity, and develop detectable circulating antibody. As described hereinbelow, the claimed TCV isolates are genetically distinct from TCV-MN. Moreover, the claimed isolates, unlike TCV-MN, produce severe thymic and bursal atrophy due to lymphocytolysis. Further, infection of turkeys with the claimed isolates resulted either in extremely high mortality, persistent infection without seroconversion,

or recovery with stunting and immune system suppression. Recovered non-carrier individuals were susceptible to re-infection.

Bovine coronaviruses (BCV) and TCV are highly related. A 99% similarity exists between the nucleocapsid and membrane proteins of BCV and TCV-MN (Dea et al., 1990). Spike ("S1") proteins of TCV-MN and BCV are also highly related, but, as described hereinbelow, the S1 gene sequences of the TCV isolates of the invention are more closely related to BCV than to TCV-MN. Naturally occurring infection of cattle with the claimed pathogenic TCV strains occurs and cattle can serve as subclinical carriers or vectors. Further support for the relatedness of BCV and TCV is shown by the cross-reactivity of polyclonal antibody to BCV and TCV. The data described herein show that TCV is a diverse viral population composed of differing pathotypes including classical TCV-MN and the more recent highly pathogenic types, and that these isolates vary in their relatedness to BCV.

Based on the relatedness of BCV to TCV, it is also envisioned that the claimed preparation or composition comprising at least one cell culture adapted TCV isolate, e.g., TCV-NC, is also useful as an immunogenic composition or vaccine in cattle, preferably to provide neutralizing and/or protective antisera to BCV. Likewise, it is envisioned that a preparation or composition comprising at least one cell culture adapted BCV isolate is useful to prepare immunogenic compositions or vaccines for turkeys so as to result in antibodies that bind to TCV. Also envisioned is a preparation or immunogenic composition comprising a plurality of enteric coronavirus isolates, e.g., a TCV isolate and a BCV isolate.

The preparation of the invention which comprises TCV or BCV, or a combination thereof, may optionally be combined with a physiologically acceptable carrier and/or optionally combined with one or more adjuvants, to yield an immunogenic composition or a vaccine. Preferably, the immunogenic composition or vaccine is injectable. The amount of isolated TCV and/or BCV in the immunogenic composition is preferably effective to actively immunize a susceptible bird, e.g., a turkey, or induce a humoral response in a mammal, e.g. a bovine.

Maternal antibodies, obtained from the sera of a female vertebrate exposed to at least one of the preparations or immunogenic compositions of the invention, or obtained from an egg from an egg-laying female vertebrate exposed to at least one of the preparations or immunogenic compositions of the invention, may be isolated and administered to animals, e.g., in feed or by injection, to provide passive immunity.

The invention further provides an isolated and purified nucleic acid molecule comprising a preselected nucleic acid sequence encoding a coronavirus polypeptide, e.g., a S1 polypeptide, a biologically active subunit, or a biologically active variant thereof, the presence of which is associated with SMT. Preferably, the preselected DNA sequence encodes a TCV S1 polypeptide. A more preferred embodiment of the invention includes a preselected DNA sequence comprising SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:30, which encodes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:31, respectively. The nucleic acid molecules of the invention are double-stranded or single-stranded.

The invention also provides an expression cassette comprising a preselected DNA sequence operably linked to a promoter functional in a host cell wherein said DNA sequence encodes a coronavirus polypeptide, a biologically active variant or subunit thereof, wherein the presence of said polypeptide in turkeys is associated with SMT. A preferred DNA sequence encodes a TCV S1 polypeptide. Such expression cassettes can be placed into expression vectors which can then be employed to transform prokaryotic or eukaryotic host cells. The expression of the preselected DNA sequence in the transformed cell results in the production of recombinant coronavirus polypeptide. The resultant recombinant polypeptide can be isolated from transformed cells.

Thus, the invention also provides isolated, purified recombinant TCV S1 polypeptide, a biologically active variant or a subunit thereof. Recombinant coronavirus S1 polypeptide, in turn, is useful to prepare polyclonal or

monoclonal antibodies. The recombinant polypeptide and antibodies thereto are useful in assays to detect the presence of coronavirus-specific antisera, or coronavirus, respectively, in vertebrates, e.g., turkey, chicken, and cattle. Thus, the polypeptide can be used as a "capture antigen" to bind to anti-polypeptide or anti-coronavirus antibodies in a sample of a physiological fluid or tissue obtained from an animal. For example, a physiological sample comprising antibodies is mixed with purified S1 polypeptide, or a preparation of isolated coronavirus, so as to yield a binary complex. The antibodies which are bound to the polypeptide or the virus are separated from the antibodies which are not bound to the polypeptide or the virus. Then the complex is detected or determined. Preferably, the complex is detected by antibodies.

Recombinant coronavirus S1 polypeptide is also useful in a vaccine or immunogenic composition which, when administered to an animal, can elicit antibodies which can inhibit or block subsequent infection of the host by the coronavirus from which the S1 gene was obtained, or a highly related coronavirus.

Further provided is an isolated and purified "antisense" nucleic acid molecule which has at least about 80%, preferably at least about 90%, and more preferably at least about 98%, nucleotide sequence complementary to SEQ ID NO:2, SEQ NO:4 or SEQ ID NO:30. These molecules can be introduced into expression cassettes, which, when expressed in a host cell can provide "anti-sense" mRNA transcripts which can alter, e.g., inhibit, TCV expression.

The nucleic acid sequences (molecules) of the present invention are useful to detect the replication or presence of the virus in infected samples, to detect related nucleic acid molecules and to amplify nucleic acid sequences, wherein said sequences fall within the scope of the present invention.

An oligonucleotide or primer of the invention preferably has at least about 80%, more preferably at least about 85%, and more preferably at least about 90%, sequence identity or homology, or sequence complementarity, to a nucleic acid molecule encoding an enteric coronavirus S1 polypeptide, such as a BCV S1 polypeptide or a TCV S1 polypeptide which is associated with SMT,

e.g., SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:30. Preferably, "anti-sense" oligonucleotides of the invention do not hybridize under stringent conditions to mouse hepatitis virus (MHV) nucleic acid sequences or other non-enteric avian coronavirus nucleic acid sequences. An oligonucleotide or primer of the invention has at least about 7 to about 50, preferably at least about 10 to about 40, and more preferably at least about 15 to about 35, nucleotides. Preferably, the oligonucleotide primers of the invention comprise at least 7 nucleotides at the 3' of the oligonucleotide primer which have at least about 85%, more preferably at least about 90%, and more preferably at least about 95%, identity or complementarity, to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:30. The oligonucleotides of the invention may also include sequences which are unrelated to nucleic acid sequences of a coronavirus S1 gene, e.g., they may encode restriction endonuclease recognition sequences. Preferred oligonucleotides include, but are not limited to, S1-1a (5′ CCG ACG TAT ACC TAA TCT TCC 3′; SEQ ID NO:9), S1-1b (5′ TGC TCA CCT ATG CCA ACT 3′; SEQ ID NO:10); S1-2a (5′GAT AAG TCG GTG CCC TCT CCA 3′; SEQ ID NO:11), and S1-2b (5′ ATG AAA GGC CGC TGA AAC AC 3′; SEQ ID NO:12). The oligonucleotides of the invention are useful in amplification reactions, and to detect the replication or presence of virus in a sample.

Also provided is a method for detecting or determining the presence of antibodies in a physiological sample obtained from an animal, which antibodies are specific for a coronavirus associated with SMT. The method comprises contacting an amount of purified coronavirus S1 polypeptide, or a clarified preparation of a cell cultured adapted isolate of coronavirus, with the sample which is suspected of comprising antibodies specific for the coronavirus, for a sufficient time to form binary complexes between at least a portion of the antibodies and a portion of the purified polypeptide or isolated virus. The presence or amount of the complexes is then determined or detected. Thus, the detection of antibody responses specific for the polypeptide or specific for the isolated virus can be used in immunoassays, e.g., ELISA-based assays, for the serodiagnosis of SMT or TCV infection in animals.

Also provided is a diagnostic kit for detecting or determining antibodies that specifically react with a coronavirus which is associated with SMT. The kit comprises packaging, containing, separately packaged, a solid phase capable of binding a polypeptide or an isolated virus preparation, and a known amount of a purified coronavirus S1 polypeptide or isolated cell culture adapted coronavirus. Preferably, the polypeptide has an amino acid sequence comprising SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:31, a variant or subunit thereof. Preferred coronavirus isolates include TCV-NC, TCV-GA, TCV-BOB, and TCV-BOA.

Also provided is a method of preventing or inhibiting SMT. The method comprises immunizing a turkey with (i) a recombinant coronavirus S1 polypeptide, and/or (ii) an isolated cell culture adapted coronavirus preparation. Preferably, the S1 polypeptide comprises SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:31, a variant or subunit thereof. Preferably, the cell culture adapted coronavirus is an isolate such as TCV-NC, TCV-GA, TCV-BOB or TCV-BOA.

Thus, the invention also provides an animal immunized with or exposed to (a) a recombinant coronavirus S1 polypeptide, and/or (b) an isolated cell culture adapted coronavirus. The immunized or exposed animal produces antibodies to the polypeptide and/or virus.

Also provided are TCV detection methods which employ nucleic acid amplification. For example, the invention provides a diagnostic method comprising contacting an amount of DNA obtained by reverse transcription of RNA from a physiological sample from a turkey at risk of, or afflicted with, SMT, or an animal exposed to said tukey, with an amount of at least two complementary oligonucleotides under conditions effective to amplify the DNA by a polymerase chain reaction so as to yield an amount of amplified DNA. At least one oligonucleotide binds specifically to a nucleic acid sequence encoding a coronavirus polypeptide, wherein the coronavirus is associated with SMT. The presence of the amplified DNA is then detected or determined. The presence of the amplified DNA is indicative of a turkey at risk of, or afflicted with, SMT, or an animal that is a carrier of the coronavirus associated with SMT.

The invention further provides a method for detecting DNA encoding an immunogenic polypeptide associated with SMT. The method comprises preparing an amount of DNA from a physiological sample, wherein the DNA is obtained by reverse transcription of RNA from said sample. The DNA is contacted with an amount of at least two oligonucleotides under conditions effective to amplify the DNA by a polymerase chain reaction so as to yield an amount of amplified DNA. At least one oligonucleotide is specific for the DNA encoding the immunogenic polypeptide. The presence of the amplified DNA is then determined or detected.

Yet another embodiment of the invention is a diagnostic kit for detecting a nucleic acid molecule that encodes at least a portion of a polypeptide which is specific for a turkey coronavirus associated with SMT in a physiological sample suspected of containing said nucleic acid molecule. The kit comprises packaging containing, separately packaged, (a) a known amount of a first oligonucleotide, wherein the oligonucleotide consists of at least about 7 to about 50 nucleotides, and wherein the oligonucleotide has at least about 80% identity to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:30, and (b) a known amount of a second oligonucleotide, wherein the oligonucleotide consists of at least about 7 to about 50 nucleotides, and wherein the oligonucleotide has at least about 80% identity to a nucleotide sequence which is complementary to SEQ ID NO:2 , SEQ ID NO:4 or SEQ ID NO:30.

Brief Description of the Figures

Figure 1. Codons for specified amino acids.

Figure 2. Tissue section from small intestine of poult with naturally occurring Spiking Mortality of Turkeys (SMT). The section was stained with hematoxylin and eosin. Bar = 30 μm.

Figure 3. Tissue section from bursa of Fabricius of poult with naturally occurring SMT which was stained with hematoxylin and eosin. The bursa has lymphoid depletion in follicular cortex (c) and medulla (m), revealing follicular epithelium (e), and increased inter-follicular space (arrow) infiltrated with lymphocytes. Bar = 30 μm.

Figure 4. Thymus tissue section from poult with naturally occurring SMT The section was stained with hematoxylin and eosin. Bar = 150 μm.

Figure 5. Electron micrograph of particles present in the feces of turkeys with naturally occurring SMT. Two percent phosphotungstic acid was employed for contrast. Bar = 50 nm.

Figure 6. Immunofluorescent staining of turkey enteritis coronavirus-(TCV) infected HRT-18 cells. The arrow indicates the location of aggregates of cells showing strong cytoplasmic fluorescence. Bar = 25 μm.

Figure 7. Transmission electron microscopy of TCV-infected HRT-18 cells. Arrows indicate coronavirus particles. Bar = 0.5 μm.

Figure 8. Transmission electron microscopy of TCV-infected HRT-18 cells. Viral particles have accumulated in the lumen of a cytoplasmic vesicle (arrow). Bar = 1.0 μm.

Figure 9. Electron micrograph of negatively stained coronavirus particles from clarified, infected cell culture supernatant. The arrow indicates surface projections typical of coronavirus. Bar = 0.1 μm.

Figure 10. Nucleotide sequence relatedness of DNA encoding coronavirus S1 polypeptide. FEV = feline enteric coronavirus (SEQ ID NO:13). HC-043 = human coronavirus strain OC43 (Genbank Accession No. L14643; SEQ ID NO:14). TGEV = porcine transmissible gastroenteritis virus (SEQ ID NO:15). Conn = Connecticut strain of Infectious Bronchitis Virus (IBV; SEQ ID NO:16). Florida = Florida strain of IBV (SEQ ID NO:17). Mass = Massachusetts strain of IBV (SEQ ID NO:18). Beaudette = Beaudette strain of IBV (SEQ ID NO:19). Gray = Gray strain of IBV (SEQ ID NO:20). JMK = JMK isolate of IBV (SEQ ID NO:21). GAV = Georgia strain of IBV (SEQ ID NO:22). CA = California strain of IBV (SEQ ID NO:23). BoCV = BOA strain of turkey coronavirus (SEQ ID NO:30). MN = Minnesota strain of TCV (SEQ ID NO:3). GA = Georgia isolate of TCV (SEQ ID NO:2). NC = North Carolina isolate of TCV (SEQ ID NO:4). BCV-L9 = bovine coronavirus strain BCV-L9 (Genbank Accession No. M64667; SEQ ID NO:24). BCV-LY138 = bovine coronavirus strain BCV-LY138 (Genbank Accession No. M646669; SEQ ID

NO:25). BCV-VACCINE = bovine coronavirus strain BCV-Vaccine (Genbank Accession No. M64668; SEQ ID NO:26). BCV-QUEB = bovine coronavirus Quebec strain (SEQ ID NO:27). BCV-F15 = bovine coronavirus strain F15 (SEQ ID NO:28). MHV2 = mouse hepatitis virus strain A59 (Genbank Accession No. M18379; SEQ ID NO:29).

Figure 11. Amino acid sequence relatedness of 21 coronavirus S1 polypeptides. FEV = feline enteric coronavirus (Genbank Accession No. X80799; SEQ ID NO:32). HC-043 = human coronavirus strain OC43 (Genbank Accession No. L14643; SEQ ID NO:33). TGEV = porcine transmissible gastroenteritis virus (Genbank Accession No. X53128; SEQ ID NO:34). Conn = Connecticut strain of IBV (SEQ ID NO:35). Florida = Florida strain of IBV (SEQ ID NO:36). Mass = Massachusetts strain of IBV (SEQ ID NO:37). Beaudette = Beaudette strain of IBV (SEQ ID NO:38). Gray = Gray strain of IBV (SEQ ID NO:39). JMK = JMK isolate of IBV (SEQ ID NO:40). GAV = Georgia strain of IBV (SEQ ID NO:41). CA = California strain of IBV (SEQ ID NO:42). BoCV = BOA strain of turkey coronavirus (SEQ ID NO:31). MN = Minnesota strain of TCV (SEQ ID NO:7). GA = Georgia isolate of TCV (SEQ ID NO:5). NC = North Carolina isolate of TCV (SEQ ID NO:8). BCV-L9 = bovine coronavirus strain BCV-L9 (Genbank Accession No. M64667; SEQ ID NO:43). BCV-LY138 = bovine coronavirus strain BCV-LY138 (Genbank Accession No. M646669; SEQ ID NO:44). BCV-VACCINE = bovine coronavirus strain BCV-Vaccine (Genbank Accession No. M64668; SEQ ID NO:45). BCV-QUEB = bovine coronavirus Quebec strain (Genbank Accession No. D00662; SEQ ID NO:46). BCV-F15 = bovine coronavirus strain F15 (Genbank Accession No. P00731; SEQ ID NO:47). MHV2 = mouse hepatitis virus strain A59 (Genbank Accession No. M18379; SEQ ID NO:48).

Figure 12. Nucleotide sequence comparison of DNA encoding the S1 polypeptide of BCV (TCV-BOA; SEQ ID NO:30), TCV-GA (SEQ ID NO:2), TCV-MN (SEQ ID NO:3), and TCV-NC (SEQ ID NO:4). The corresponding amino acid sequences are SEQ ID NO:31, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8, respectively.

Detailed Description of the Invention

Definitions

As used herein, the terms "isolated and/or purified" refer to *in vitro* preparation, isolation and/or purification of a nucleic acid molecule, polypeptide or peptide of the invention, so that it is not associated with *in vivo* substances.

The term "isolated" with respect to a viral isolate or strain of the invention refers to a virus preparation that was obtained by *in vitro* culture and propagation, or alternatively, by *in vivo* passage and subsequent *in vitro* isolation. Preferably, an isolated virus preparation of the invention is substantially free of other infectious agents.

As used herein, "a coronavirus S1 polypeptide or peptide" is preferably a TCV polypeptide having an amino acid sequence comprising SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:31, or a biologically active subunit thereof. A "variant" coronavirus S1 polypeptide is a polypeptide having an amino acid sequence which has at least about 80%, preferably at least about 90%, and more preferably at least about 95%, but less than 100%, identity or homology to SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:31, or a biologically active subunit thereof. A variant polypeptide or peptide of the invention may include amino acid residues not present in the corresponding wild type S1 polypeptide or peptide, as well as internal deletions relative to the corresponding wild type S1 polypeptide or peptide.

Preferably, the polypeptides, peptides and viral preparations of the invention are biologically active. For example, biologically active coronavirus S1 polypeptides, peptides and variants thereof falling within the scope of the invention have at least about 1%, preferably at least about 10%, more preferably at least about 50%, and even more preferably at least about 90%, the activity of the polypeptide comprising SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:31. The activity of a coronavirus polypeptide or peptide, or a viral isolate, can be measured by methods well known to the art including, but not limited to, the ability of the polypeptide, peptide or virus to be bound by antibodies specific for

TCV, e.g., specific for the TCV S1 protein, the ability of the polypeptide, peptide or virus to elicit a sequence-specific immunologic response when the polypeptide is administered to an animal such as a bird or a mammal, e.g., rabbit, goat, bovine, sheep, rat or mouse, or the correlation of the presence of the virus, or a virus having the polypeptide, and SMT. Preferably, the immunologic response is a humoral response, i.e, antibody response, directed to a particular epitope on the polypeptide, peptide or virus. More preferably, the presence of antibodies specific for that epitope correlates with the SMT infection status of the organism.

The biological activity of a viral preparation of the invention can be measured by methods known to the art, some of which are described hereinbelow. For example, a coronavirus isolate of the invention is propagated in culture, e.g., in HRT-18 cells. The isolates of the invention induce cytopathic effect (cpe) *in vitro* in sensitive cells, and/or have hemagluttination activity (Dea et al., 1991). The isolate binds anti-TCV antibodies, as determined by assays such as IFA or a neutralization assay (Example 4). The biological activity of a virus preparation of the invention *in vivo* can be determined by administering the virus preparation to turkeys so as to induce SMT, or to animals, e.g., turkeys or chickens, so as to elicit a specific immune response, e.g., a response which results in immunization or a virus-specific humoral response.

An isolated "variant" nucleic acid molecule of the invention is a nucleic acid molecule which has at least 80%, preferably at least about 90%, and more preferably at least about 95%, but less than 100%, nucleotide sequence homology or identity to the nucleotide sequence of the corresponding wild type nucleic acid molecule, e.g., a DNA sequence comprising SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:30. Moreover, a variant nucleic acid molecule of the invention may include nucleotide bases not present in the corresponding wild type nucleic acid molecule, as well as internal deletions relative to the corresponding wild type nucleic acid molecule.

I. Identification of Coronavirus Isolates Falling Within the Scope of the Invention

A. Sources of Turkey or Bovine Coronaviruses

Turkeys having or having been exposed to turkeys having SMT, as well as farm animals residing on farms having or which had affected turkeys, e.g., cows or chickens, are reservoirs of TCV and BCV isolates of the invention. Preferred tissue sources of enteric coronaviruses include feces, duodenum, jejunum, ileum, bursa (for avian sources), lymph node (for non-avian sources, e.g., mammals), spleen, and thymus from these animals.

Further sources include turkeys or embryos thereof, chickens or embryos thereof, bovines and cell lines that have been experimentally infected with the viruses, as well as insects and wildlife which harbor the viruses (e.g., deer, birds, and rodents). "Carriers" of virus are organisms in which the virus replicates but does not cause significant disease.

B. Adaptation to Cell Culture, and Propagation and Isolation of Virus

Coronaviruses falling within the scope of the invention may be adapted to grow in intestinal cultures from any species, e.g., rat, pig, dog and human. A preferred cell culture is a permanent cell line, e.g. HRT-18 cells. Also preferred are cultures which exhibit cytopathic effect upon infection. However, the viruses may also be maintained and propagated *in vivo* in adult turkeys, poults or embryos, chickens, cattle and wild life (see above).

HRT-18 cells are cultured in MEM with 3-10% fetal bovine sera at 37°C in 5% CO_2. Once a monolayer is established (approximately 24-48 hours after 8.12×10^5 cell/ml HRT-18 cells are seeded onto a surface of 35 cm^2 or greater), the media is removed. Clarified and filtered (0.45 micron) inoculum is then added to the monolayer at 37°C, 5% CO_2 for one hour. After incubation, MEM is added and the infected cells incubated for 5-7 days. The cells are then frozen at -70°C and thawed. The thaw is clarified and the supernatant is preferably filtered prior to addition to uninfected monolayers of HRT-18 cells.

Isolated coronaviruses falling within the scope of the invention include viruses which bind to anti-TCV antibodies and/or anti-BCV antibodies, e.g., as

determined by immunofluorescent assays (IFA) or neutralization assays, and/or have hemagluttination (HA) properties, and which induce SMT upon inoculation into susceptible animals. If the isolate has been propagated *in vitro* or *in vivo* in a species which is not susceptible, the isolate may require several back passages before pathogenicity is observed.

C. Sources and Isolation of Nucleic Acid Molecules of the Invention

Sources of nucleotide sequences from which the present nucleic acid molecules, i.e., molecules which encode coronavirus polypeptides, can be derived include nucleic acid from any eukaryotic source, preferably a farm animal, known or believed to be naturally or experimentally infected by a coronavirus of the invention, i.e., TCV associated with SMT or BCV useful to induce antibodies in turkeys which bind to TCV. The source may be cellular or acellular (sera or plasma which contain the virus, or acellular egg components such as yolk and albumin) in origin. Preferably, the source is an *in vitro* source, i.e., cell cultures infected with the virus.

A nucleic acid molecule encoding a coronavirus S1 polypeptide can be identified and isolated using standard methods, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY (1989). For example, reverse transcriptase-polymerase chain reaction (RT-PCR) can be employed to isolate and clone coronavirus S1 genes. "PCR" refers to a procedure or technique in which amounts of a preselected fragment of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195.

In RT-PCR, sequence information from the ends of the region of interest or beyond is employed to identify and synthesize oligonucleotide primers comprising at least 7-8 nucleotides. These primers will be identical or similar in sequence to opposite strands of the template to be amplified. In general, PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, and the like. See generally Mullis et al., Cold Spring Harbor Symp. Quant. Biol., 51, 263 (1987); Erhlich, ed., PCR Technology, (Stockton Press, NY, 1989).

Primers are made to correspond to nucleic acid molecules encoding highly conserved regions of polypeptides or to nucleotide sequences which were identified and compared to generate the primers, e.g., by a sequence comparison of other coronavirus S1 genes. Preferably, at least two primers are prepared, one of which is predicted to anneal to the antisense strand, and the other of which is predicted to anneal to the sense strand of a nucleic acid molecule which encodes the coronavirus S1 polypeptide. The products of each PCR reaction are separated via an agarose gel and all consistently amplified products can be gel-purified and cloned directly into a suitable vector, such as a plasmid vector. The resultant plasmids are subjected to restriction endonuclease and dideoxy sequencing of double-stranded plasmid DNAs.

Thus, RNA is isolated from a cellular source believed to be infected with a coronavirus isolate of the invention, or supernatants from cells infected *in vitro* with the isolate. The RNA is reverse transcribed to form a single strand cDNA. The cDNA is then mixed with primers, as described above, and PCR is performed. The resultant DNA fragments encode all or a portion of a gene encoding a coronavirus S1 polypeptide. These fragments can be further characterized by sequence analysis, or by expression in host cells and subsequent screening for binding to antibodies from an animal that has been infected by the same or a related coronavirus. DNA fragments that have at least some sequence identity or homology to other coronavirus S1 polypeptides, or which encode polypeptides that are immunoreactive with above-mentioned antibodies, can be subcloned into a suitable vector and used as probes to identify other nucleic acid sequences encoding all or a portion of a coronavirus S1 polypeptide.

As used herein with respect to a nucleic acid molecule or polypeptide, the terms "isolated and/or purified" refer to *in vitro* isolation of a nucleic acid molecule or polypeptide molecule from its natural cellular environment, and from association with other components of the cell, such as nucleic acid or protein, so that it can be sequenced, replicated, and/or expressed. For example, "isolated nucleic acid encoding coronavirus S1 polypeptide" is RNA or DNA containing greater than 7, preferably 15, and more preferably 20 or more,

sequential nucleotide bases. The sequential nucleotide bases encode a biologically active coronavirus S1 polypeptide, preferably a polypeptide associated with SMT, or a fragment thereof, or a biologically active variant coronavirus S1 polypeptide, or a fragment thereof. The DNA or RNA is complementary to the non-coding strand, or complementary to the coding strand, of RNA from the virus which causes, or is associated with, SMT and not complementary to the RNA or DNA from related organisms which do not cause, or are not associated with, SMT, or hybridizes to said RNA or DNA and remains stably bound under stringent conditions.

Thus, the RNA or DNA is "isolated" in that it is free from at least one contaminating nucleic acid with which it is normally associated in the natural source of the RNA, and the isolated RNA or DNA is preferably substantially free of any other viral or eukaryotic RNA or DNA. The phrase "free from at least one contaminating source of nucleic acid with which it is normally associated" includes the case where the nucleic acid is introduced into a host or cell but, prior to introduction, was isolated from a viral preparation, infected cells or an infected host, or obtained from *in vitro* expression, e.g., an expression cassette. An example of isolated RNA or DNA encoding a polypeptide of the invention is RNA or DNA that encodes a biologically active, i.e., immunogenic, coronavirus S1 polypeptide sharing at least about 80%, preferably at least about 90%, sequence identity with the S1 polypeptide encoded by the nucleic acid molecules of Figure 12 ("NC", "GA" and "BO").

As used herein, the term "recombinant nucleic acid" or "preselected nucleic acid," e.g., "recombinant nucleic acid sequence or segment" or "preselected nucleic acid sequence or segment" refers to a nucleic acid, e.g., to DNA, that has been derived or isolated from any appropriate cellular or acellular source, that may be subsequently chemically altered *in vitro*, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in a non-recombinant viral genome. An example of a preselected DNA "derived" from a source, would be a DNA sequence that is identified as a useful fragment, and which is then

chemically synthesized in essentially pure form. An example of such RNA "isolated" from a source would be a RNA sequence that is obtained from said source by chemical means, e.g., by the use of reagents which remove viral capsids without affecting the integrity of viral nucleic acid, so that it can be further manipulated, e.g., reverse transcribed and amplified, for use in the invention by the methodology of genetic engineering. For example, RT-PCR of coronavirus nucleic acid may be employed to obtain amplified fragments having S1 genes. These fragments can be separated by polyacrylamide or agarose gel electrophoresis, identified by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. See Lawn et al., Nucleic Acids Res., 9, 6103 (1981), and Goeddel et al., Nucleic Acids Res., 8, 4057 (1980). Therefore, "preselected DNA" includes completely synthetic DNA sequences, semi-synthetic DNA sequences, DNA sequences isolated from biological sources, and DNA sequences derived from RNA, as well as mixtures thereof.

As used herein, the term "derived" with respect to a RNA molecule means that the RNA molecule has complementary sequence identity to a particular DNA molecule.

D. Variants of the Nucleic Acid Molecules of the Invention

Nucleic acid molecules encoding amino acid sequence variants of a coronavirus S1 polypeptide can be prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of a coronavirus S1 polypeptide.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing amino acid substitution variants of a coronavirus S1 polypeptide. This technique is well known in the art as described by Adelman et al., DNA, 2, 183 (1983). Briefly, coronavirus S1-specific DNA is altered by hybridizing an

oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the S1 polypeptide. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the coronavirus S1 DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al., Proc. Natl. Acad. Sci. U.S.A., 75, 5765 (1978).

The DNA template can be generated by those vectors that are either derived from bacteriophage M13 vectors (the commercially available M13mp18 and M13mp19 vectors are suitable), or those vectors that contain a single-stranded phage origin of replication as described by Viera et al., Meth. Enzymol., 153, 3 (1987). Thus, the DNA that is to be mutated may be inserted into one of these vectors to generate single-stranded template. Production of the single-stranded template is described in Sections 4.21-4.41 of Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, N.Y. 1989).

Alternatively, single-stranded DNA template may be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the coronavirus S1 DNA, and the

other strand (the original template) encodes the native, unaltered sequence of the coronavirus S1 DNA. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for peptide or polypeptide production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutations(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thiodeoxyribocytosine called dCTP-(aS) (which can be obtained from the Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101.

For example, a preferred embodiment of the invention is an isolated and purified DNA molecule comprising a preselected DNA segment encoding a

coronavirus S1 polypeptide, the expression of which in turkeys is associated with SMT, such as a preselected DNA encoding SEQ ID NO:6, e.g., a DNA comprising SEQ ID NO:2, or a DNA having nucleotide substitutions which are "silent" (see Table 1). That is, when silent nucleotide substitutions are present in a codon, the same amino acid is encoded by the codon with the nucleotide substitution as is encoded by the codon without the substitution. For example, valine is encoded by the codon GTT, GTC, GTA and GTG. A variant of SEQ ID NO:2 at the fifteenth codon in a S1 polypeptide (GTT in SEQ ID NO:2) includes the substitution of GTC, GTA or GTG for GTT.

Other "silent" nucleotide substitutions in SEQ ID NO:2 which can encode polypeptide having SEQ ID NO:6 can be ascertained by reference to Table 1 and page D1 in Appendix D in Sambrook et al., Molecular Cloning: A Laboratory Manual (1989), as well as Table 1 hereinbelow. Nucleotide substitutions can be introduced into DNA segments by methods well known to the art, some of which are described above. See, also, Sambrook et al., *supra*. Likewise, nucleic acid molecules encoding other S1 polypeptides may be modified in a similar manner. Thus, nucleic acid molecules encoding at least a portion of SEQ ID NO:7 or SEQ ID NO:8, or the complement thereto, may be modified so as to yield nucleic acid molecules of the invention having silent nucleotide substitutions, or to yield nucleic acid molecules having nucleotide substitutions that result in amino acid substitutions (see polypeptide or peptide variants hereinbelow).

E. Preparation of Chimeric Expression Cassettes and Their Introduction into Host Cells

To prepare expression cassettes for transformation herein, the recombinant or preselected nucleic acid sequence or segment may be circular or linear, double-stranded or single-stranded. A preselected DNA sequence which encodes an RNA sequence that is substantially complementary to a mRNA sequence encoding a coronavirus S1 polypeptide is typically a "sense" DNA sequence cloned into a cassette in the opposite orientation (i.e., 3′ to 5′ rather than 5′ to 3′). Generally, the preselected nucleic acid sequence or segment is in

the form of chimeric DNA, such as plasmid DNA, that can also contain coding regions flanked by control sequences which promote the expression of the preselected DNA present in the resultant cell line.

As used herein, "chimeric" means that a vector comprises DNA from at least two different species, or comprises DNA from the same species, which is linked or associated in a manner which does not occur in the "native" or wild type of the species.

Aside from preselected DNA sequences that serve as transcription units for a coronavirus S1 polypeptide, or portions thereof, a portion of the preselected DNA may be untranscribed, serving a regulatory or a structural function. For example, the preselected DNA may itself comprise a promoter that is active in prokaryotic or eukaryotic cells, or may utilize a promoter already present in the genome that is the transformation target. Such promoters in eukaryotes include the CMV promoter, as well as the SV40 late promoter and retroviral LTRs (long terminal repeat elements), although many other promoter elements well known to the art may be employed in the practice of the invention.

Other elements functional in the host cells, such as introns, enhancers, polyadenylation sequences and the like, may also be a part of the preselected DNA. Such elements may or may not be necessary for the function of the DNA, but may provide improved expression of the DNA by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the DNA as desired to obtain the optimal performance of the transforming DNA in the cell.

"Control sequences" is defined to mean DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotic cells, for example, include a promoter, and optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

"Operably linked" is defined to mean that the nucleic acids are placed in a functional relationship with another nucleic acid sequence. For example, DNA

for a presequence or secretory leader is operably linked to DNA for a peptide or polypeptide if it is expressed as a preprotein that participates in the secretion of the peptide or polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

The preselected DNA to be introduced into the cells further will generally contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of transformed cells from the population of cells sought to be transformed. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a co-transformation procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide-resistance genes, such as *neo, hpt, dhfr, bar, aroA, dapA* and the like. See also, the genes listed on Table 1 of Lundquist et al. (U.S. Patent No. 5,848,956).

Reporter genes are used for identifying potentially transformed cells and for evaluating the functionality of regulatory sequences. Reporter genes which encode for easily assayable proteins are well known in the art. In general, a reporter gene is a gene which is not present in or expressed by the recipient organism or tissue and which encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Preferred genes include the chloramphenicol acetyl transferase gene (cat) from Tn9 of *E. coli*, the beta-glucuronidase gene (gus) of the *uidA* locus of *E. coli*, and the luciferase

gene from firefly *Photinus pyralis*. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells.

In particular, vectors such as baculovirus vectors, *E. coli* or *Salmonella* vectors, or pox virus vectors are useful to express the polypeptide of the invention.

The general methods for constructing recombinant DNA which can transform target cells are well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the DNA useful herein. For example, J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (2d ed., 1989), provides suitable methods of construction.

The recombinant DNA can be readily introduced into the host cells, e.g., mammalian, bacterial, e.g., *E. coli* or *Salmonella*, fungal, yeast or insect cells by transfection with an expression vector comprising a nucleic acid molecules of the invention by any procedure useful for the introduction into a particular cell, e.g., physical or biological methods, to yield a transformed cell having the recombinant DNA, preferably stably integrated into its genome, so that the nucleic acid molecules, sequences, or segments of the present invention are expressed by the host cell.

Physical methods to introduce a preselected DNA into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Biological methods to introduce the DNA of interest into a host cell include the use of DNA and RNA viral vectors. The main advantage of physical methods is that they are not associated with pathological or oncogenic processes of viruses. However, they are less precise, and can result in multiple copy insertions, random integration, disruption of foreign and endogenous gene sequences, and unpredictable expression. Viral vectors can be derived from poxviruses, herpes simplex virus I, retroviruses, adenoviruses and adeno-associated viruses, and the like.

Bacterial vectors, useful to express fusion proteins encoded by at least a portion of an S1 gene operably linked to another gene in bacteria, e.g., in *E. coli*

or *Salmonella*, can also be employed. The transformed bacteria, administered in vehicles such as water or feed, can populate the host organism's intestine where the recombinant S1 polypeptide, preferably a fusion polypeptide comprising at least a portion of an S1 polypeptide is expressed and excreted. This method of administration can give rise to cellular and/or mucosal immunity.

As used herein, the term "cell line" or "host cell" is intended to refer to well-characterized homogenous, biologically pure populations of cells. These cells may be eukaryotic cells that are neoplastic or which have been "immortalized" *in vitro* by methods known in the art, as well as primary cells, or prokaryotic cells. The cell line or host cell is preferably of mammalian origin, but cell lines or host cells of non-mammalian origin may be employed, including avian, plant, insect, yeast, fungal or bacterial sources. Preferred mammalian and avian cells are intestinal in origin.

"Transfected" or "transformed" is used herein to include any host cell or cell line, the genome of which has been altered or augmented by the presence of at least one preselected nucleic acid sequence, e.g., DNA, which DNA is also referred to in the art of genetic engineering as "heterologous DNA," "recombinant DNA," "exogenous DNA," "genetically engineered," "non-native," or "foreign DNA," wherein said DNA was isolated and introduced into the genome of the host cell or cell line by the process of genetic engineering. The host cells of the present invention are typically produced by transfection with a DNA sequence in a plasmid expression vector, a viral expression vector, or as an isolated linear DNA sequence. Preferably, the transfected DNA is a chromosomally integrated recombinant DNA sequence, which comprises a gene encoding a coronavirus S1 polypeptide or its complement.

To confirm the presence of the preselected DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; or "biochemical" assays, such as detecting the presence or absence of a S1 polypeptide, e.g., by immunological means (ELISAs and Western blots).

F. Polypeptides, Peptides, and Variants Thereof

The present isolated, purified coronavirus S1 polypeptides, peptides, or variants thereof, can be synthesized *in vitro*, e.g., by the solid phase peptide synthetic method or by recombinant DNA approaches (see above). When a coronavirus S1 polypeptide of the invention is expressed in a recombinant cell, it is necessary to purify the polypeptide from other recombinant cell proteins or polypeptides to obtain preparations that are substantially homogenous as to the S1 polypeptide. For example, the culture medium or lysate can be centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The S1 polypeptide may then be purified from the soluble protein fraction. Alternatively, the S1 polypeptide may be purified from the insoluble fraction, i.e., refractile bodies (see, for example, U.S. Patent No. 4,518,526), if necessary. S1 polypeptide may be purified from contaminant soluble or membrane proteins and polypeptides by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an anion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; or ligand affinity chromatography, and the like.

If expressed as a fusion polypeptide, the fusion polypeptide may be purified by methods specific for the non-S1 polypeptide portion of the polypeptide. For example, if the fusion polypeptide is a glutathione-S transferase (GST) fusion polypeptide, GST 4B beads may be employed to purify the fusion polypeptide.

S1 polypeptide, variant S1 polypeptide, or a biologically active subunit thereof, can also be prepared by *in vitro* transcription and translation reactions. A S1 polypeptide expression cassette can be employed to generate S1 gene-specific transcripts which are subsequently translated *in vitro* so as to result in a preparation of substantially homogenous S1 polypeptide, variant S1 polypeptide, or a biologically active subunit thereof. The construction of vectors for use *in*

vitro transcription/translation reactions, as well as the methodologies for such reactions, are well known to the art.

The solid phase peptide synthetic method is an established and widely used method to prepare peptides and polypeptides, which is described in the following references: Stewart et al., Solid Phase Peptide Synthesis, W. H. Freeman Co., San Francisco (1969); Merrifield, J. Am. Chem. Soc., 85 2149 (1963); Meienhofer in "Hormonal Proteins and Peptides," ed.; C.H. Li, Vol. 2 (Academic Press, 1973), pp. 48-267; and Bavaay and Merrifield, "The Peptides," eds. E. Gross and F. Meienhofer, Vol. 2 (Academic Press, 1980) pp. 3-285. These polypeptides or peptides can be further purified by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an anion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; or ligand affinity chromatography.

Once isolated and characterized, derivatives, e.g., chemically derived derivatives, of a given S1 polypeptide or peptide can be readily prepared. For example, amides of the S1 polypeptide, peptide or variants thereof of the present invention may also be prepared by techniques well known in the art for converting a carboxylic acid group or precursor, to an amide. A preferred method for amide formation at the C-terminal carboxyl group is to cleave the peptide from a solid support with an appropriate amine, or to cleave in the presence of an alcohol, yielding an ester, followed by aminolysis with the desired amine.

Salts of carboxyl groups of a polypeptide, peptide or variant of the invention may be prepared in the usual manner by contacting the polypeptide or peptide with one or more equivalents of a desired base such as, for example, a metallic hydroxide base, e.g., sodium hydroxide; a metal carbonate or bicarbonate base such as, for example, sodium carbonate or sodium bicarbonate; or an amine base such as, for example, triethylamine, triethanolamine, and the like.

N-acyl derivatives of an amino group of the polypeptide, peptide or variant of the invention may be prepared by utilizing an N-acyl protected amino acid for the final condensation, or by acylating a protected or unprotected peptide. O-acyl derivatives may be prepared, for example, by acylation of a free hydroxy peptide or peptide resin. Either acylation may be carried out using standard acylating reagents such as acyl halides, anhydrides, acyl imidazoles, and the like. Both N- and O-acylation may be carried out together, if desired.

Formyl-methionine, pyroglutamine and trimethyl-alanine may be substituted at the N-terminal residue of the polypeptide, peptide or variant. Other amino-terminal modifications include aminooxypentane modifications (see Simmons et al., Science, 276, 276 (1997)).

In addition, the amino acid sequence of the polypeptide or can be modified so as to result in a polypeptide or peptide variant. The modification includes the substitution of at least one amino acid residue in the peptide for another amino acid residue, including substitutions which utilize the D rather than L form, as well as other well known amino acid analogs.

One or more of the residues of the polypeptide or peptide can be altered, so long as the resultant variant is biologically active. Conservative amino acid substitutions are preferred--that is, for example, aspartic-glutamic as acidic amino acids; lysine/arginine/histidine as basic amino acids; leucine/isoleucine, methionine/valine, alanine/valine as hydrophobic amino acids; serine/glycine/alanine/threonine as hydrophilic amino acids.

Conservative substitutions within the scope of the invention include those shown in Table 1 under the heading of exemplary substitutions. More preferred substitutions are under the heading of preferred substitutions. After the substitutions are introduced, the variants are screened for biological activity.

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Amino acid substitutions falling within the scope of the invention, are, in general, accomplished by selecting substitutions that do not differ significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic; trp, tyr, phe.

The invention also envisions polypeptide or peptide variants with non-conservative substitutions. Non-conservative substitutions entail exchanging a member of one of the classes described above for another.

Acid addition salts of the polypeptide, peptide or variant thereof or of amino residues of the polypeptide, peptide or variant may be prepared by contacting the polypeptide, peptide, variant or amine thereof with one or more equivalents of the desired inorganic or organic acid, such as, for example, hydrochloric acid. Esters of carboxyl groups of the polypeptides or peptides may also be prepared by any of the usual methods known in the art.

G. Detection of Coronavirus S1 Antibodies

The present invention further relates to diagnostic assays for use in veterinary medicine. For diagnosis of SMT, the presence of antibodies to the S1 polypeptide of TCV or to the corona virus associated with SMT in animal serum is determined. Many types of test formats, as one skilled in the art will recognize, can be used. Such tests include, but are not limited to, IFA, RIA, RIST, ELISA, agglutination and hemagglutination. The diagnostic assays can be performed using standard protocols such as those described by Magnarelli et al., J. Clin. Microbiol., 20, 81 (1984); Craft et al., J. Infect. Dis., 149, 789 (1984); Enguall et al., Immunochemistry, 8, 871 (1971); and Russell et al., J. Infect. Dis., 149, 465 (1984).

Specifically, a diagnostic assay of the present invention can be constructed by coating on a surface (i.e., a solid support) for example, a plastic bead, a microtitration plate or a membrane (e.g., nitrocellulose membrane), all or a unique portion of the S1 polypeptide (natural or synthetic), or an isolated virus preparation, and contacting it with the serum or other physiological fluid taken from an animal suspected of having a TCV infection or SMT. Following removal of the physiological fluid, any antibody bound to the immobilized S1 polypeptide or virus preparation (the antigen) can be detected, preferably by reacting the binary antibody-antigen complexes with a "detection antibody",

which detection antibody comprises a detectable label or a binding site for a detectable label. Suitable detectable labels are enzymes, fluorescent labels or radiolabels.

In another embodiment of the diagnostic assay of the present invention, all or a unique portion of the S1 polypeptide or isolated virus preparation is bound to an inert particle of, for example, bentonite, polystyrene or latex. The particles are mixed with serum from an animal in, for example, a well of a plastic agglutination tray. The presence or absence of antibodies in the animal's serum is determined by observing the settling pattern of the particles in the well.

In a further embodiment of the diagnostic assay of the present invention, the presence or absence of TCV in a serum sample is detected. Antibodies specific for the S1 polypeptide or a unique antigenic portion thereof, or antibodies specific for the virus, can be coated onto a solid surface such as a plastic and contacted with the serum sample. After washing, the presence or absence of TCV bound to the fixed antibodies is detected by addition of a labeled (e.g., fluorescently labeled) antibody specific for TCV or the S1 polypeptide.

H. Dosages, Formulations and Routes of Administration of the Viral Preparations, Nucleic Acid Molecules and Polypeptides of the Invention

The virus preparations (including cells infected with the coronavirus isolates of the invention), nucleic acid molecules, polypeptides or peptides of the invention are preferably administered to an animal, e.g., a turkey or bovine, so as to result in an immune response specific for the virus or a related virus (e.g., administration of TCV to cattle may protect cattle against BCV infection), polypeptide, including the polypeptide encoded by the nucleic acid molecules of the invention, or peptide. These compounds and compositions can be administered to avians and mammals for veterinary use, such as for use with domestic or farm animals. In general, the dosage of virus required for efficacy will range from about 0.1 cc containing about 10^3 virions to about 1.0 cc containing about 10^3 virions, preferably about 0.1 cc containing about 10^4 virions to about 1.0 cc containing about 10^4 virions, and more preferably about 0.1 cc

containing 10^5 virions to about 1.0 cc containing 10^5 virions, although other dosages may provide beneficial effects. Methods to determine the titer of a viral stock include the determination of $TCID_{50}$ and hemaglutinin titer (HA titer).

For polypeptides and nucleic acid molecules of the invention, the dosage required is about 0.01 μg to about 300 mg, preferably about 0.1 μg to about 200 mg, and more preferably about 5 μg to about 100 mg, although other amounts may prove efficacious.

Dosages within these ranges can be administered via bolus doses or via a plurality of unit dosage forms, until the desired effects have been obtained. The amount administered will vary depending on various factors including, but not limited to, the specific immunogen chosen, the age of the animal, live versus killed virus (for virus or infected cell inocula), and the route of inoculation.

Preferably, the administration is to a turkey so as to result in an immune response which inhibits or prevents SMT, or to a chicken or bovine so as to result in the production of antibodies to the virus, polypeptide or peptide employed as an immunogen. Both local and systemic administration is contemplated. Systemic administration is preferred.

Also envisioned is the administration of maternal antibody, which antibody is obtained from a female animal exposed to a virus preparation, virus infected cells, a nucleic acid molecule or polypeptide of the invention. For example, a hen is vaccinated with at least one of the immunogenic compositions of the invention. The hen then provides passive immunity to progeny through the transfer of maternal antibody to the embryo. Alternatively, an egg-laying animal, e.g., a chicken, may be immunized and the eggs from that animal collected. Antibody is recovered from the eggs and then administered to susceptible animals, e.g., turkeys, to provide passive protection. Preferably, the turkeys are subsequently exposed to live or killed virus, or other compositions of the invention, to provide active protection.

Administration of sense or antisense nucleic acid molecules may be accomplished through the introduction of cells transformed with an expression cassette comprising the nucleic acid molecule (see, for example, WO 93/02556)

or the administration of the nucleic acid molecule (see, for example, Felgner et al., U.S. Patent No. 5,580,859, Pardoll et al., Immunity, 3, 165 (1995); Stevenson et al., Immunol. Rev., 145, 211 (1995); Molling, J. Mol. Med., 75, 242 (1997); Donnelly et al., Ann. N.Y. Acad. Sci., 772, 40 (1995); Yang et al., Mol. Med. Today, 2, 476 (1996); Abdallah et al., Biol. Cell, 85, 1 (1995)). Pharmaceutical formulations, dosages and routes of administration for nucleic acids are generally disclosed, for example, in Felgner et al., *supra*.

The viral compositions may be administered as live, modified-live (attenuated) or inactivated virus, or optionally administered as a combination of attenuated, inactivated, and/or live virus, or in combination with a nucleic acid molecule of the invention, a polypeptide or peptide of the invention, or any combination thereof. Moreover, the administration of more than one immunogenic agent of the invention to an animal may occur simultaneously or at different times. The virus may be inactivated by formalin, beta-propriolactone or ethylenimines.

Typically, immunogenic compositions are prepared for injection or infusion, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection or infusion may also be prepared. The preparation may also be emulsified. The active ingredient can be mixed with diluents, carriers or excipients which are physiologically acceptable and compatible with the active ingredient(s). Suitable carriers can be positively or negatively charged or neutral avridine-containing liposomes, oil emulsions; live-in-oil; killed-in-oil, water-in-oil; $Al(OH)_3$; oil emulsion with terpene oils squalene or squalene; or aqueous. Suitable diluents and excipients are, for example, water, saline, PBS, glycerol, or the like, and combinations thereof. In addition, if desired, the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH-buffering agents, and the like.

Such compositions are conventionally administered parenterally, by injection, for example in birds, either intravenously, intramuscular injection to breast, lung or thigh, subcutaneous or via the beak, as well as by spraying the

animals and their environment, e.g., their housing or yard. The administration of maternal antibody is preferably in feed or water. Nucleic acid, polypeptide and virus of the invention are preferably administered in feed, water or by spraying. Formulations which are suitable for other modes of administration include suppositories, cloaca, insufflated powders or solutions, eye drops, nose drops, intranasal aerosols, and oral formulations, e.g., introduced into drinking water. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of alkylcelluloses, mannitol, dextrose, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. Thus, these compositions can take the form of solutions, suspensions, tablets, pills, hard or soft gelatin capsules, sustained-release formulations such as liposomes, gels or hydrogels; or powders, and can contain about 10% to about 95% of active ingredient, preferably at about 25% to about 70%.

One or more suitable unit dosage forms comprising the virus preparations, nucleic acid molecules, polypeptides or peptides of the invention, which may optionally be formulated for sustained release. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known to pharmacy. Such methods may include the step of bringing into association the therapeutic agent with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system.

For administration of a virus preparation of the invention to turkeys, chickens or bovines, the preparation may be administered at dosages of at least about 10^1 to about 10^2 virions, preferably about 10^2 to about 10^3 virions and more preferably about 10^3 to about 10^4 virions, although other dosages may provide beneficial results. A unit dose of the vaccine is preferably administered parenterally, e.g., by subcutaneous or intramuscular injection.

To prepare an immunogenic composition comprising a coronavirus S1 polypeptide, peptide or variant thereof, the purified S1 polypeptide, peptide or variant can be isolated as described hereinabove, lyophilized and stabilized. The

polypeptide antigen may then be adjusted to an appropriate concentration, optionally combined with a suitable carrier and/or suitable vaccine adjuvant, and preferably packaged for use as a vaccine. Suitable adjuvants include, but are not limited to, surfactants, e.g., hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dioctadecyl-n'-N-bis(2-hydroxyethyl-propane di-amine), methoxyhexadecyl-glycerol, and pluronic polyols; polanions, e.g., pyran, dextran sulfate, poly IC, polyacrylicacid, carbopol; peptides, e.g., muramyl dipeptide, dimethylglycine, tuftsin, oil emulsions, alum, and mixtures thereof. Finally, the immunogenic product may be incorporated into liposomes for use in a vaccine formulation, or may be conjugated to polysaccharides or other polymers.

For administration of polypeptide, peptide or nucleic acid to turkeys, chickens or bovines, the amount administered may be at dosages of at least about 0.01 μg to about 300 mg, preferably about 0.1 μg to about 200 mg, more preferably about 5 μg to about 100 mg, and even more preferably at least about 10 μg to about 50 mg of polypeptide, although other dosages may provide beneficial results. The absolute weight of the polypeptide, peptide or nucleic acid included in a given unit dosage form of vaccine can vary widely, and depends upon factors such as the species, age, weight and physical condition of the animal considered for vaccination, as well as the method of administration, e.g., feed, water or spray. Such factors can be readily determined by the veterinarian employing animal models or other test systems which are well known to the art. A unit dose of a polypeptide vaccine is preferably administered parenterally, e.g., by subcutaneous or by intramuscular injection. The polypeptides or peptides of the invention may also be conjugated or linked to an immunogenic protein, such as KLH or albumin, to enhance their immunogenicity.

Other factors to be considered include whether the animal is a breeder or a production animal. Production animals may be individually exposed to an immunogenic composition of the invention, preferably virus, either live or killed, preferably at 1 day of age (doa) by ingestion, inhalation or injection. A breeder

animal, however, may be exposed at 1 doa with killed virus as well as at multiple later points in time, preferably by interspersing live and killed virus inocula.

The invention will be further described with reference to the following Examples.

Example 1

Experimental reproduction of SMT in isolation facilities

Materials and Methods

Two commercial turkey houses meeting the criteria for SMT were identified. From each house, litter (110 kg) and moribund poults (n = 50) were collected. Collected litter was stored at 4°C. Moribund poults were killed by rapid cervical disarticulation and necropsied. Samples of duodenum, jejunum, ileum, bursa, spleen, and thymus were collected aseptically and homogenized in a chilled sterile blender with equal volumes of 4°C phosphate buffered saline to make a SMT-organ homogenate. This homogenate was stored at 4°C.

An organ homogenate was also prepared from clinically normal poults from the same geographic production area (non-SMT organ homogenate). Samples of small intestine, bursa, thymus, bone marrow, brain, lung, liver, spleen, kidney, sciatic nerve, heart, and puboischiofemoralis muscle were collected at necropsy for histopathology and fixed by immersion in 10% neutral buffered formalin. Tissues were embedded in paraffin blocks, and 5 micron sections prepared. The resultant slides were stained with hematoxylin and eosin employing routine methods, and then examined using light microscopy.

For direct electron microscopic identification of viral particles, samples of intestinal and bursal contents were collected from selected poults and mixed with equal volumes of distilled deionized water. This mixture was applied to a copper grid coated with carbon and 3% polyvinyl formvar in ethylene dichloride (Electron Microscopy Sciences, Fort Washington, PA), blotted dry and negatively stained by floating grids on a drop of 2% phosphotungstic acid at pH 7.0 for 3 minutes. Grids were then examined in a transmission electron microscope at 80 KEV. For immunoelectron microscopic demonstration of coronaviral reactivity, rabbit-origin polyclonal antiserum reactive with Quebec-

and Minnesota-origin TCV (provided by Dr. S. Dea, University of Quebec, Canada) was mixed with equal volumes of intestinal or bursal content, allowed to stand at room temperature for 30 minutes, mounted on grids, and examined as described above.

Poults used to reproduce SMT as described below were unvaccinated, uninjected, white production 1 day-old hen turkeys from a commercial hatchery. They were fed routine unmedicated University of Georgia turkey starter and given access to clean water *ad libitum*. There was no SMT apparent in maternal flocks of these poults or in hatchmates placed in commercial turkey houses.

For transmission via litter in floor pens, 25 kg of litter collected from affected houses was spread into floor pens with concrete floors, HEPA-filtered positive-pressure air supply, and a single central heat lamp per pen. One hundred 1 day-old poults were placed on this litter (n = 50 per pen). An additional one hundred 1 day-old poults were placed on new litter in separate isolation rooms as negative controls. Ambient room temperature was maintained at 30°C, surface litter temperature under the center of the heat lamp at 40°C, and litter moisture at 40% water by weight. This experiment was repeated in the same floor pens with an equal number of poults.

For transmission via organ homogenates in isolation chambers, 0.5 cc of SMT-organ homogenate was administered by oral gavage to each of 120 poults at 1 day-of-age. An additional 120 poults were administered 0.5 cc of the non-SMT organ homogenate and served as vehicle controls. Another 120 1 day-old poults were not gavaged, placed in identical isolation units, and served as negative controls. Poults (n = 15 per replicate, 8 replicates per treatment) were housed in wire-floored positive-pressure modified Horsfall isolation units. Paper was spread over the wire floor on days 1 to 2 and then removed. Ambient air temperature in the isolators was 30°C on days 1 to 2, 35°C on days 4 to 7, 32°C on days 8 to 14, and 30°C on days 14 to 21. This experiment was repeated in the same isolation units with an equal number of poults.

For both methods of transmission, the number of birds dying each day, totaled for each week, was recorded. Body weights and feed conversions were

measured once weekly. Poults dying naturally were necropsied within 8 hours of death. All surviving poults were killed at 21 days of age by rapid cervical disarticulation and necropsied immediately. Samples of small intestine, bursa, and thymus were collected from each poult at necropsy and sections for histopathology prepared and examined as described above. Samples of intestinal and bursal content from dead poults were collected on post-exposure days 5, 14, and 21, prepared as described above, and examined using direct and immune electron microscopy.

Results

Histopathology samples collected from the naturally occurring SMT cases had lesions in the small intestine, bursa, and thymus which were evident using light microscopy. There was severe shortening of villi (Figure 2), fusion, and hyperplasia of crypts. Villus atrophy was less severe in the duodenum. Enterocytes on villus tips were sloughing into the intestinal lumen. Bursal sections had moderate to severe variation in follicle size with increased interfollicular connective tissue and space interpreted as edema (Figure 3). Follicular cortices were 2-4 lymphocytes thick, follicular epithelium was hyperplastic, and medullary portions of follicles were moderately depleted of lymphocytes. Thymic sections had severe cortical thinning with medullary tissue comprising over 90% of the thymic mass (Figure 4). Direct electron microscopy of intestinal and bursal contents revealed a pleomorphic 70-140 nm diameter particle with short 5 to 10 nm surface projections over its profile (Figure 5). Identical particles were adhered into large aggregates by mixing with polyclonal antisera containing anti-coronaviral antibody.

In experimental transmission in floor pens (exposed from 0 to 21 days of age; Table 2), mortality was over 1% per day on days 4 through 10. In survivors, body weights were suppressed and feed conversions were increased. Atrophy of intestinal villi, bursal follicles, and thymic cortices in poults dying on day 5 were identical to those found in the naturally occurring disease. Lesions became worse over time in poults surviving longer. These lesions were still pronounced in poults surviving to 21 days of age. Direct electron microscopy

revealed 70-140 nm diameter particles in intestinal and bursal content identical to those seen in naturally occurring cases. These particles were seen on day 5, but not on days 14 or 21. Results in the replication of this experiment were morphologically and statistically the same as the first trial.

Table 2

	Mortality (# dead/# alive)		BW (g)		FC (g feed/g BW)	
Age (days)	SMT	Control	SMT	Control	SMT	Control
0	0/100	0/100	53.5	54.3	N/A	N/A
7	15/100	1/100	71.6	150.8	2.54	1.27
14	23/85	0/99	189.7	333.1	4.82	1.73
21	7/62	0/99	241.2	561.4	5.07	1.51

Mortality = weekly mortality
BW = mean body weight
FC = weekly feed conversion
SMT = Spiking Mortality of Turkeys
Control = new litter

In experimental transmission in modified Horsfall isolation units (exposed once at 1 day of age; Table 3), mortality was over 1% per day on days 4 through 10. In survivors, body weights were suppressed and feed conversions were worsened. Histologic lesions were identical to those in poults with naturally occurring SMT and in those with SMT experimentally transmitted in floor pens. Direct electron microscopy again revealed 70-140 nm diameter particles on day 5, but not on days 14 or 21 post-exposure. Results in the replication of this experiment were morphologically and statistically the same as the first trial.

Table 3

Age (days)	Mortality (# dead/# alive)			BW (g)			FC (g feed/g BW)		
	SMT	Non-SMT	Control	SMT	Non-SMT	Control	SMT	Non-SMT	Control
0	0/120	0/120	0/120	52.3	52.0	52.1	N/A	N/A	N/A
7	32/120	1/120	2/120	62.4	123.7	121.4	6.67	1.25	1.24
14	12/88	1/119	0/118	189.7	341.2	333.1	4.23	1.57	1.59
21	7/76	0/118	0/118	241.2	570.9	561.4	5.07	1.60	1.63

Mortality = weekly mortality
BW = mean body weight
FC = weekly feed conversion
SMT = organ homogenate from turkeys with SMT
Non-SMT = Non-SMT organ homogenate
Control = unexposed

No lesions or viral particles were seen in poults placed on new litter, those given non-SMT organ homogenate, or those left unexposed.

Discussion

Severe enteritis consistent with SMT is positively correlated statistically with enteric coronavirus infection, but not with other enteric virus infections (Goodwin et al., 1995). SMT has been experimentally reproduced (Brown et al., 1996b). The histologic lesions described hereinabove are identical to those produced by turkey enteric coronavirus infection (Dea et al., 1991; Gonder et al., 1976), rotaviral infection in turkeys (Yason et al., 1987), and enteritis secondary to exposure to turkey litter contaminated with rotavirus, cryptosporidium, and other infectious agents (Perry et al., 1991).

The 70-140 nm particle in the small intestinal and bursal content of the naturally occurring and experimentally transmitted SMT cases is morphologically consistent with turkey enteric coronaviruses (Naqi et al., 1972; Dea et al., 1988; Dea et al., 1991; Ritchie et al., 1973). The 70-140 nm particle has been isolated and antigenically confirmed as a coronavirus by fluorescent antibody analysis (Garcia et al., 1996).

Major outbreaks of coronaviral enteritis in turkeys have occurred previously in Minnesota (Patel et al., 1977) and Quebec (Dea et al., 1988). The recent severe outbreak of SMT may be due to re-emergence of a previously described turkey enteric coronavirus with increased pathogenicity due to additional exacerbating factors. Alternately, recent outbreaks of SMT may be due to naturally occurring recombination of pre-existing coronaviruses of other species and their acquisition of enteropathogenic features in turkeys (Dea et al., 1991).

Example 2

In vitro decontamination techniques for SMT

Materials and Methods

Coronavirus-containing organ homogenates were prepared as previously described (Example 1). Briefly, poults were experimentally inoculated with a coronavirus-containing SMT organ homogenate, killed, and samples of intestines, bursa, spleen, and thymus were collected aseptically, homogenized in a sterile blender with equal volumes of 4°C phosphate buffered saline, and divided into 7 equal aliquots.

To assess the effects of pH, one aliquot was adjusted to pH 2.0 by addition of 38.0% HCl (Baker, Phillipsburg, NJ), and another adjusted to pH 12.0 by addition of 98.7% NaOH (Mallinckrodt, Paris, KT). To assess the effects of temperature, one aliquot was held in a heat block at 57°C. To assess the effects of drying, one aliquot was lyophilized. To assess the effects of chemical agents, 37% formaldehyde solution (Baker, Phillipsburg, NJ) was added to one aliquot to achieve a final concentration of 1.0% and 99.2% NaCl (Baker, Phillipsburg, NJ) and was added to another aliquot to achieve a final concentration of 5.0% by weight. One aliquot was left untreated to serve as a positive control. The samples were maintained at room temperature, except the 57°C aliquot, for 5 hours.

Unvaccinated, uninjected, white production 1 day-old hen turkeys (n=240) from a commercial hatchery were divided into 8 equal treatments with 2 replicates for each treatment. Poults in a replicate (n=15) were placed in an

individual wire-floored electrically heated modified Horsfall isolation unit, fed routine unmedicated University of Georgia turkey starter, and given access to clean water *ad libitum*. Poults in each treatment were administered 1 ml of one of the 7 aliquots of inocula by oral gavage. Poults in one treatment were not gavaged and served as negative controls.

The number of birds dying each day, totaled for each week, was recorded by replicate and summarized by treatment. Body weights and feed consumption were measured and feed conversions calculated once weekly by treatment. Mean body weights were analyzed by treatment using Turkey-Kramer HSD (version 3.1 of JMP®) ($p < 0.05$). Samples of intestinal and bursal content were collected on post-exposure days 5, 14, and 21 and examined using direct electron microscopy and immunoelectron microscopy using anti-coronavirus antisera as described in Example 1. All poults dying during the trial were necropsied within 8 hours of death, and all survivors were killed at 21 days of age and necropsied.

Results

To identify techniques useful to decontaminate commercial turkey houses, the physicochemical lability of the infectious nature of intestinal inocula was determined. Poults receiving untreated inoculum (positive controls), or inocula exposed to either NaCl, pH 2, pH 12, 56°C, or lyophilization, had severely depressed body weights and poor feed conversions at 7, 14, and 21 days post-inoculation (Tables 4 and 5). Droppings contained coronavirus on day 5, but not on day 14 or 21. Cumulative mortality was greater than 60% by day 14 in each of these treatments except for pH 2. Lesions in dead poults consisted of jejunal distention with watery clear fluid, and bursal and thymic atrophy.

Table 4

Treatment	Mean body weight (g)[a] / Weekly Mortality Days post inoculation[b] 0	7	14	21
Positive Control	51.8/0	68.2/7	102.9/9	229.7/10
NaCl	51.7/0	62.6/9	111.8/14	* /7
pH 2.0	51.8/0	92.4[a]/5	141.4/6	247.8/6
pH 12	51.3/0	67.8/7	106.2/11	227.6/6
Formaldehyde	52.0/0	117.0[a]/0	228.3[a]/0	389.2[a]/0
57°C	51.6/0	76.1/7	122.5/11	266.0/5
Lyophilization	51.6/0	67.2/8	107.0/10	217.6/7
Unexposed	51.8/0	118.0[a]/0	235.2[a]/1	344.0[a]/0

* Mortality = 100%.
[a] = Mean weights with different superscripts are statistically different at $p < 0.05$ using Tukey-Kramer HSD.
[b] = Poults inoculated at 1 day of age.

Poults that were not gavaged (unexposed) and those receiving formalin-treated inoculum developed no clinically evident disease, gross lesions, or depressions of body weight or feed conversion. No coronavirus was detected in their feces using electron microscopy and mortality was 3% or less in these treatments.

Discussion

In previous outbreaks of coronaviral enteritis in turkeys, strict decontamination and poultry depopulation eliminated the disease from most farms (Patel et al., 1977). In recent outbreaks, these practices have been less than successful. Turkey enteric coronaviruses can be carried subclinically by cattle and chickens (Brown et al., 1995; Brown et al., 1996a; Dea et al., 1991). This may be one cause for failure of decontamination of affected turkey farms. Other possible causes include lack of compliance with decontamination protocols, persistence in local wildlife vectors, increased environmental stability of this virus, or viral resistance to routine chemical disinfectants.

Turkey enteric coronavirus associated with Bluecomb Disease in Minnesota had variable response to heat and pH (Deshmukh et al., 1969). In contrast, in homogenates produced from experimentally infected embryos, the Minnesota isolate was stable at pH 3, stable for 1 hour at 50°C without magnesium chloride exposure, but killed at this temperature with magnesium chloride (Deshmukh et al., 1974).

In an effort to mimic natural exposure, organ homogenates from infected poults were employed as inocula. The ability of these inocula to produce disease and induce coronaviral fecal shedding was resistant to pH 12, heating to 57°C, lyophilization, and NaCl exposure. Treatment at pH 2 partially ameliorated mortality and negative effects on weight depression and feed conversion to levels intermediate between the positive and negative controls. These results suggest intestinal coronaviruses in organ homogenates, and possibly, on naturally affected turkey farms, are protected by cell-association, or by mixing with other fecal or tissue components. The only decontamination technique that mitigated all the effects of coronavirus-containing inoculum was formaldehyde.

Table 5

	Weekly feed conversion (g feed/g gain)			
	Days post inoculation[a]			
Treatment	0-7	7-14	14-21	0-21
Positive Control	7.46	*	*	*
NaCl	4.29	*	*	*
pH 2.0	1.63	2.73	2.96	2.88
pH 12	4.77	*	*	*
Formaldehyde	1.15	1.74	1.68	1.42
57°C	2.20	*	*	*
Lyophilization	5.46	*	*	*
Unexposed	1.20	1.66	1.60	1.53

* = Mortality over 60%, no feed conversion calculated.
[a] = Poults inoculated at 1 day of age.

Example 3

Ultrastructural Changes and Indirect Immunofluorescence of Two Turkey Enteric Coronaviruses Isolates

Materials and Methods

Specimens. Intestinal contents and intestinal tracts from one to two week old turkey poults with mild to severe diarrhea were obtained from commercial flocks in North Carolina (UGA-APN) and Georgia (UGA-APG). Specimens were prepared as previously described (Dea et al., 1988). Briefly, samples were clarified by centrifugation at 5,000 x g for 10 minutes at 4°C. Supernatants were filtered using 0.45 μm filters and frozen at -70°C.

The reference Minnesota strain of turkey coronavirus (MN-TCV) has been previously described (Dea et al., 1989a; Dea et al., 1989b; Dea et al., 1989c; Dea et al., 1991).

Virus propagation. Isolation and propagation of viruses was performed by two methods. In one method, clarified clinical specimens of UGA-APN and UGA-APG isolates and the reference Minnesota strain of TCV were inoculated (0.2-0.3 ml) into the amniotic cavity of 22 to 24 day old embryonated turkey eggs (Adams et al., 1970; Dea et al., 1985; Deshmukh et al., 1973; Pomeroy, 1980) obtained from a source known to be free from common pathogens of turkeys and with no history of coronaviral enteritis. Negative control turkey embryos were similarly inoculated with deionized distilled water. After inoculation, all eggs were incubated at 37°C for three days, then necropsied, and gross lesions were recorded. Embryo intestines were harvested and homogenized in tryptose phosphate broth (TPB) (Dea et al., 1985). The homogenates were clarified by centrifugation at 5,000 x g for 10 minutes and by filtration using 0.45-μm filters. The resultant supernatants were used for subsequent embryo inoculations.

The other method employed to isolate and propagate viruses was the human rectal adenocarcinoma cell line HRT-18 (Dea et al., 1985), which was prepared in culture flasks and on Leighton coverslips (Dea et al., 1989e; Dea et al., 1985; Dea et al., 1989b; Dea et al., 1991). The HRT-18 cell line was

propagated in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA), 50 µg of gentamicin/ml (Atlanta Biologicals, Atlanta, GA), and 10 U of bovine crystallized trypsin/ml (Sigma Chemical, St. Louis, MO). Confluent monolayers were inoculated with the clarified UGA-APN and UGA-APG clinical specimens or with the reference Minnesota strain of TCV. Five to six days postinoculation (PI), the monolayers were frozen and collected (Pomeroy, 1980) for electron microscopy (EM) analysis, hemagglutination (HA) activity and indirect immunofluorescence (IFA). Non-inoculated HRT-18 monolayers were used as negative controls and were processed similarly.

Antisera. Rabbit hyperimmune sera reactive with purified egg-adapted or tissue-culture-adapted reference Minnesota strain has been previously described (Dea et al., 1990a; Dea et al., 1989e; Dea et al., 1989d; Dea et al., 1985; Dea et al., 1989b).

Indirect immunofluorescence (IFA). The reference Minnesota strain and the UGA-APN and UGA-APG inoculated HRT-18 cell monolayers were checked daily for cytopathic effect (CPE). At ninety-six hours PI, the monolayers developed CPE, and the cells were frozen and thawed twice. The samples were then centrifuged at 5,000 x g for 15 minutes (Lyerla et al., 1979). The pellets were placed in glass slides using sterile loops, air dried and fixed in cold acetone (-29°C) for 15 minutes, rinsed three times in PBS and air dried. The smears were processed for indirect staining using the hyperimmune rabbit anti-TCV serum (dilution 1:5), and incubated at 37°C for 15 minutes, rinsed in phosphate buffered saline (PBS) and air dried. A fluorescein-conjugated mouse anti-rabbit IgG (Sigma Immunochemicals, St. Louis, MO), dilution 1:5, was added and incubated for 15 minutes at 37°C. Smears were rinsed in PBS, and air dried (Lyerla et al., 1979). Three different negative controls were prepared similarly using un-inoculated HRT-18 cells and negative rabbit serum.

Electron microscopy (EM). Specimens were processed in a similar fashion as mentioned above. Aliquots (0.5 ml) from clarified fecal specimens from poults with diarrhea and clarified infected-cell culture fluid were negatively

stained with 2% sodium phosphotungstate (pH 7) for direct EM (Bozzola et al., 1991; Dea et al., 1989e; Dea et al., 1989d).

Confluent cell monolayers in Leighton tubes were inoculated with 0.2 to 0.5 ml of clarified viral suspensions of the Minnesota TCV (MN-TCV), UGA-APN or UGA-APG isolates. After incubation for 30 minutes at 37°C, the cell cultures were given maintenance medium (with 3% FBS, Atlanta Biologicals, Atlanta, GA) containing 10 U of bovine crystallized trypsin/ml (Sigma Chemical, St. Louis, MO). Leighton tubes were checked daily for CPE. The coverslips were fixed with 2% glutaraldehyde for 2 hours and washed in TBS (0.2 M Tris-buffered saline, Sigma, St. Louis, MO). A second fixation was performed with 2% osmium tetroxide and then dehydrated in ethanol series of progressively increasing concentrations up to absolute ethanol, sectioned and stained with toluidine blue. Thick sections (1.0 μ) were checked for cellular morphology and CPE by light microscopy and affected monolayers were further processed.

Briefly, tissues were sectioned (600 Å thick), mounted on 200-mesh formvar carbon-coated nickel grids and stained with methanolic uranyl acetate (5% uranyl acetate, Ernest F. Fullam, Inc., Lathan, NY) for one minute, jet washed in deionized water, post-stained in Reynolds lead citrate (2.66% lead citrate, Baker, Phillipsburg, NJ; 3.52% sodium citrate, Baker, Phillipsburg, NJ; and 0.64% sodium hydroxide, Fisher Scientific, Norcross, GA) for eight minutes and washed in deionized water (Bozzola et al., 1991). The cell monolayers on the Leighton coverslips were examined by transmission electron microscopy (TEM) at 24 hours, 48 hours, 72 hours, and 96 hours PI.

Results

Both isolates, the UGA-APN and UGA-APG of TCV, were tested for their ability to propagate in embryonating eggs of turkeys inoculated via the amniotic cavity. Turkey embryos necropsied at three to four days PI had intestines distended up to twice the normal size and were filled with fluid and gas. Coronaviral particles were visualized by EM from the intestinal contents of these embryos. Supernatants of these intestinal contents were inoculated into

HRT-18 cells which then developed CPE. No gross lesions were found in the negative control turkey embryos.

CPE induced by the TCV isolates was first apparent on the fourth or fifth passage. The first noticeable CPE in infected cell monolayers was the appearance of small, discrete syncytia and round, large, granular and refractile cells at 48 hours PI. By 72 hours PI, syncytia cells were more frequent and contained up to four to five nuclei. At 96 hours PI, there was detachment of the affected monolayer, formation of syncytia cells containing up to six nuclei, and increased cytoplasmic granulation. Monolayers were not completely destroyed. All viral isolates produced similar CPE. The negative control monolayers did not exhibit CPE.

Freeze-thaw treatments did not completely destroy the cells and many cells maintained their normal morphology and architecture. Coronaviral antigens were first detected at 24 hours PI, when diffuse cytoplasmic fluorescence was observed in individual infected cells. Staining was more intense in the cellular cytoplasm of isolated, or groups of, infected cells at 72 hours and 96 hours PI (Figure 6). All three turkey viral isolates were positive using IFA. Throughout the experiment, specific staining was never observed in control preparations and coronaviral antigens were not detected in the remaining intact nuclei of infected cells.

Inoculated HRT-18 cell monolayers were mostly intact and in fair condition at 48 and 72 hours PI. In contrast, at 96 hours PI these monolayers were severely disrupted and cells were fragmented.

Typical coronaviral particles were detected using transmission electron microscopy (TEM). Viral particles were found from 48 hours to 96 hours PI. At 48 hours and 72 hours, the coronaviral particles were seen in a Golgi apparatus forming face or a smooth transitional region of the rough endoplasmic reticulum, previously characterized as a pre-Golgi compartment or vesicle (Avers, 1986; Holmes et al., 1995). Later, newly synthesized virions were also present in the lumina of the endoplasmic reticulum and Golgi apparatus (Holmes et al., 1995). Some particles were budding from the endoplasmic reticulum membrane (Figure

7). These particles were moderately pleomorphic but mostly spherical in shape, enveloped, and approximately 60 to 200 nm in diameter (Figure 8).

Typical particles had a central electron lucent or dense matrix 60 to 70 nm in diameter, surrounded by a double-layered band 17 to 30 nm in thickness (Figure 9). The inner layer of this band consisted of darkly stained, knob-like long surface projections and a less obvious inner fringe of shorter surface projections (Dea et al., 1989e; Dea et al., 1989d; Dea et al., 1989c; Dea et al., 1990b; Holmes et al., 1995). The light, more evenly stained outer layer had no distinguishing features. Surface projections, particularly the shorter fringe, were difficult to appreciate in the thin section preparations.

At 96 hours PI, cytoplasmic vesicles were markedly enlarged, contained numerous coronaviral particles, and in many cases had ruptured onto the cellular surface.

Discussion

Previously, coronaviruses had been observed using electron microscopy in the feces of PEMS/SMT-infected turkeys, but propagation and isolation of these viruses had proven difficult. However, two PEMS/SMT-associated TCV strains were propagated in HRT-18 cells and in turkey-embryonated eggs. Evidence of viral replication in UGA-APN and UGA-APG TCV HRT-18 infected cells included the production of typical coronaviral particles as observed by electron microscopy, and the production of time-dependent increases in fluorescent staining for coronaviral antigens.

The ultrastructural properties of the TCV isolates and the findings in thin sections indicated that the UGA-APN and UGA-APG strains share major properties such as size, morphology and site of replication with the reference Minnesota strain of the *Coronaviridae* family (Dea et al., 1989d; Dea et al., 1985; Dea et al., 1989c; Dea et al., 1990). Moreover, the observations that coronaviral particles have two different types of surface projections concur with the morphological features previously described for mammalian hemagglutinating coronaviruses (bovine coronavirus, porcine hemagglutinating encephalomyelitis virus) (Dea et al., 1989a; Dea et al., 1990b; Verbeek et al.,

1991) and for avian coronaviruses (turkey enteric coronavirus) (Dea et al., 1990b; Dea et al., 1991; Verbeek et al., 1991). In addition, IFA confirmed that the reference Minnesota TCV is related to these two coronaviruses. The strong positive reaction observed in the UGA-APN and UGA-APG TCV-infected cells and the absence of labeling in the negative controls confirms that the viruses that replicated in the HRT-18 cells shared antigenic determinants with the reference Minnesota strain.

Previous reports demonstrated a 99% similarity between the amino acid sequence of the N and M proteins of TCV and bovine coronavirus (BCV) (Dea et al., 1990b; Verbeek et al., 1991), although polyclonal chicken anti-BCV did not neutralize TCV. In contrast, polyclonal rabbit anti-BCV (Nebraska) neutralized TCV. Likewise, polyclonal rabbit anti-TCV neutralized BCV (Nebraska). Furthermore, a recent report describing the isolation of coronavirus antigenically indistinguishable from BCV from wild ruminants suggests that wild ruminants could constitute a reservoir for BCV (Tsunemitsu et al., 1995). These findings suggest the possibility of persistence of coronavirus infection of turkeys in the environment in domestic and wildlife reservoirs or vectors.

Example 4

***In vitro* anti-viral activity of acute and convalescent serum and yolk**

Materials and methods

Neutralization assay. One hundred microliters of serum (heat inactivated and filtered through a 0.45 micron filter) was serially diluted (1:2) in a 96 well plate. One hundred microliters of virus, $10^{3.5}$/ml of UGA-APN, was added to the wells. The mixtures were incubated for 40 minutes at 25°C. One hundred microliters of each dilution was transferred to a 96 well microtiter plate with an HRT-18 cell monolayer. These plates were incubated in 5% CO_2 at 37°C for 5 days. Endpoints (cpe) were determined microscopically.

Results

Chickens were inoculated twice intramuscularly (0.5 cc in breast and 0.5 cc in thigh at 22 and 23 weeks of age) with killed UGA-APN (exposed to 0.01%

formaldehyde for 24 hours on a stir plate). Serum from these hyperimmunized chickens (collected at 24 weeks of age) neutralized UGA-APN.

Turkeys were inoculated three times intramuscularly (0.5 cc in the breast at 6, 7, and 8 weeks of age) with killed UGA-APN (exposed to 0.01% formaldehyde for 24 hours on a stir plate). Serum from these turkeys neutralized UGA-APN (endpoint of 8-1024). Serum from turkeys inoculated subcutaneously (i.e., in the neck at 11 and 12 weeks of age) had a virus neutralization endpoint of < 2-16.

Serum from experimentally exposed turkeys, inoculated with live UGA-APN orally at 1 day of age (1 cc of 10^6 $TCID_{50}$ /ml), the neutralization endpoint was < 2. A similar endpoint was observed in naturally exposed turkeys positive by IFA (endpoint < 2). Yolk obtained from turkeys which were orally administered TCV showed that the virus remained active *in vivo* (Tables 6 and 7). In contrast, yolk from hyperimmunized chickens (killed UGA-APN, 2 intramuscular injections) had a neutralization titer of 1024.

Hygromycin B (5 mM in low pH media) had no TCV neutralizing activity *in vitro*.

In summary, acute and convalescent sera and yolk from naturally occurring and experimental cases of SMT/PEMS in turkey and chicken were examined for antiviral activity using virus neutralization assays in HRT-18 cell cultures. The TCV isolates employed to quantify TCV neutralizing activity were adapted to HRT-18. Virus neutralizing antibody was generated by intramuscular immunization of chickens and turkeys. Neutralizing antibody was also found in the yolk of hyperimmunized hens. However, neutralizing antibody was not found after oral exposure of poults to TCV.

Table 6

TREATMENT	Mean Body Weights (g) 1 day old[A]	7 days old[A]	14 days old[A]	20 days old[A]
Negative Control	48	138	300	510
Positive Control	48	111	241	463
TCV .5 kg/1000 lbs	49	128	255	461
TCV 2 kg/1000 lbs	49	128	250	455

[A] Weights by age with different superscripts are statistically different ($p < 0.05$ Tukey-Kramer HSD).
TCV = UGA-APN isolate of turkey intestinal coronavirus grown in HRT-18 cells.
Mortality in all groups was less than 6%.

Table 7

TREATMENT	Mean Feed Conversion (g feed / g bird) 1-7 day old	7-14 day old	14-20 day old	1-20 day old
Negative Control	1.12	1.35	1.67	1.36
Positive Control	1.49	1.47	1.75	1.58
TCV .5 kg/1000 lbs	1.15	1.55	1.70	1.52
TCV 2 kg/1000 lbs	1.18	1.58	1.46	1.44

TCV = UGA-APN isolate of turkey intestinal coronavirus grown in HRT-18 cells.

Example 5

Clinical and Histopathological Findings in Turkey Poults Inoculated with Bovine Enteric Coronaviruses

Cell adapted (HRT-18 cells) bovine-origin intestinal coronaviruses (Nebraska BCV, and bovine-origin TCV "A" , i.e., TCV-BOA, and "B", i.e., TCV-BOB) were inoculated orally (1 cc of 10^6 $TCID_{50}$/ml) into 1 day-old turkey poults and backpassed ten times. For each passage, effects on clinical signs, body weight (Table 8), feed conversion (Table 9) and pathologic changes were determined. Three to five days postinoculation, the inoculated birds were

depressed, dehydrated and many had a moderate to severe diarrhea, but had no significant gross lesions. Poor body weight gain and feed conversion was noted in inoculated turkeys.

Gross pathology included emaciation and diarrhea, small intestinal dilatation, distension of the ceca, mild bursal atrophy and mild thymic atrophy. Histopathological examination of thymus, bursa of Fabricius, small and large intestine revealed moderate to severe bursa atrophy and villus atrophy of the duodenum, jejunum, and ileum. Moderate lymphocytic enteritis and moderate to severe atrophy of cortex and medulla of the thymus was also observed. Marked differences were found in the body weight and feed conversion between control and inoculated birds.

These results indicated that the inoculation of turkeys with cell cultured adapted bovine-origin coronaviruses can result in SMT-like symptoms in exposed birds.

Table 8

Body Weights (one week)

Passage	Controls	Inoculated	
	$\bar{y}$	$\bar{y}$	
4	142.0	115.0	114.0
5	113.4	98.5	99.8
6	128.3	99.4	101.2
8	143.2	121.4	119.0
9	116.1	91.2	94.4

Mean of each inoculated group was significantly different from control of same passage level (Tukey-Kramer and Student's t, alpha = 0.05).

Table 9

Feed Conversion (one week)

P	Control	Inoculated	
5	1.0	1.6	3.0
6	1.5	2.4	1.9
7	1.4	1.8	1.8
8	1.7	2.4	2.1
9	1.5	2.3	1.9

Example 6

Propagation, titration, and virus neutralization of turkey enteric coronaviruses in HRT-18 cells

Materials and Methods

Propagation. Sources: Intestines from poults (UGA-APN and UGA-APG) and feces from cows (UGA-APBOB). HRT-18 cells were grown in MEM with 10% FBS, 5% CO_2 at 37°C, and seeded in plates or flasks at a concentration of 8.12×10^5 cells/ml.

Titration. 96 well microtiter plates were seeded with HRT-18 cells (3.56×10^5/ml) and allowed to form a monolayer. Ten-fold dilutions of clarified virus were added after 24 hours. Plates were incubated in 5% CO_2 at 37°C for 5 days and endpoints read microscopically.

Neutralization assay. The neutralization assay was performed essentially as described above (Example 4).

Results

TCV isolates were titrated by serial dilution of virus in HRT-18 cells to endpoints as visualized by cytopathic effect (cpe). A virus neutralization (VN) assay was employed to determine the neutralizing titer of sera taken from infected turkeys, vaccinated turkeys, and suspected carriers of TCV.

At passages 1-5, none of the cultures showed cytopathic effect (cpe). At passage 6, UGA-APN and UGA-APG infected cultures showed cpe after 5 to 7 days. By passage 8, UGA-APBOB infected cultures were cytopathic after 5 to 7 days. By passage 15, all infected cultures had cpe after 2 days. Viral titers were

UGA-APN p16 = $10^{6.5}$/ml, UGA-APG p13 = $10^{6.5}$/ml, UGA-APBOB p13 = $10^{5.5}$/ml, and TCV-MN p18 = $10^{5.5}$/ml.

The results for neutralization analyses were as follows. Heat inactivated, filtered sera from hyperimmunized rabbits (immunized with killed UGA-APN, 4 times intramuscularly at weekly intervals with 1 cc of virus) had a virus neutralization (VN) titer of 2,560 - 10,240. For experimentally infected turkeys (live SMT homogenate, 1 time orally at 1 doa), the VN titer was < 2 at 1, 2 and 3 weeks post-infection). For vaccinated turkeys (UGA-APN, 2 live oral administrations of 10^6 $TCID_{50}$/ml at 6 and 7 weeks of age, 1 cc of killed virus administered subcutaneously at 8 weeks, and bled at 10 weeks) had VN titer of < 2 to 32 while suspect carrier cows (originated from SMT farms) had VN titer of 8 to 2,048.

Chicken IBV (infectious bronchitis virus) antiserum (SPAFAS origin) with a VN titer to IBV Mass of 1024 had a VN titer to TCV of 16. SPAFAS origin sera with a neutralization index (NI) of 5.9 to IBV/072 had a VN titer to TCV of less than 2. SPAFAS sera with a NI to IBV/Ark of 6.1 had a VN titer to TCV of less than 4. Bovine BCV antiserum (NVSL origin) had a VN titer to BCV of 2,560 and to TCV of 2,048.

Thus, the virus neutralization assay described above detected virus neutralizing antibodies to the homologous TCV. Moreover, while anti-BCV antibodies neutralized TCV, there was little cross-reactivity of TCV isolates with IBV antibodies.

Example 7

Sequence comparison of the S1 gene of turkey enteric coronavirus with bovine enteric coronavirus and avian and mammalian coronaviruses

Coronaviruses are enveloped viruses and the envelope contains the viral glycoproteins M, S ("spike protein"), and HE. The spike protein ("S1") binds to a specific host cell receptor and may induce fusion of viral envelope with the cell membrane. Neutralizing antibodies are produced against S1. Among coronaviruses, there is diversity in nucleotide sequence and molecular mass of

S1. Moreover, there are several hypervariable regions in S1, which include deletions and insertions.

To compare the S1 gene sequence of TCV field isolates with avian and mammalian coronaviruses, the S1 gene of TCV isolates UGA-APBOA, UGA-APG, MN-TCV, and UGA-APN was amplified using RT-PCR. RNA was extracted from UGA-APBOA, UGA-APG, MN-TCV, and UGA-APN infected HRT-18 cell cultures using phenol/chloroform extraction. RNA isolation was performed essentially according to Stern and Kennedy (1980) using the Rnaid Kit (BIO 101, Inc., Vista, CA). Two hundred units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) was used in a reverse transcriptase reaction (1 hour at 37°C) to obtain single-stranded cDNA from TCV RNAs using protocols such as those described in Sambrook et al. (1989). A primer pair (e.g., SEQ ID NO:9 and SEQ ID NO:10 or SEQ ID NO:11 and SEQ ID NO:12), Taq DNA polymerase (5 µl/reaction), and PCR buffer (Promega Corp., Madison, WI; 50 mm KCl, 10 mM Tris HCl, pH 9.0 at 25°C), 0.1% Triton X-100 and 3 mm $MgCl_2$) were then added to individual samples. The mixture was then subjected to 30 cycles at 95°C for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes. The PCR products were separated by gel electrophoresis on 1% agarose gels, stained with ethidium bromide and visualized under UV light. The primer pair SEQ ID NO:9 and SEQ ID NO:10 yielded a PCR product of 717 bp while SEQ ID NO:11 and SEQ ID NO:12 yielded a PCR product of 1070 bp. The amplified products were sequenced using an automated sequencer. The S1 nucleotide sequence and corresponding amino acid sequence of the amplified S1 genes were compared to other avian, bovine and mammalian coronavirus S1 sequences using Dnasis (Figure 11).

The TCV S1 gene is about 700 bases longer than the avian IBV S1 gene. Phylogenetic analysis of the deduced amino acid sequence reveals a 98% similarity with most of the BCV isolates analyzed, and < 20% similarity with avian bronchitis isolates (Figures 9 and 10). Sequence analysis indicated that UGA-APBOA and UGA-APG are the same virus.

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The invention is not limited to the exact details shown and described, for it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention defined by the claims.

WHAT IS CLAIMED IS:

1. An isolate of turkey coronavirus obtained from a turkey having spiking mortality of turkeys or obtained from an animal exposed to said turkey, which isolate is adapted to cell culture.

2. The isolate of claim 1 which comprises UGA-APN, UGA-APG, UGA-APBOB, UGA-APBOA, or a combination thereof.

3. A vaccine or immunogenic composition comprising the isolate of claim 1.

4. The vaccine of claim 3 which comprises live turkey coronavirus.

5. The vaccine of claim 3 which comprises killed turkey coronavirus.

6. The vaccine of claim 3 which further comprises a pharmaceutically acceptable carrier.

7. A preparation of isolated antibody obtained from an animal exposed to the vaccine or immunogenic composition of claim 3, wherein the preparation comprises antibodies that bind the isolate of claim 1.

8. An isolate of bovine coronavirus which, when administered to a turkey, results in the production of antibodies that bind to turkey coronavirus.

9. A vaccine or immunogenic composition comprising the isolate of claim 8.

10. The vaccine of claim 9 which comprises live bovine coronavirus.

11. The vaccine of claim 9 which comprises killed bovine coronavirus.

12. A preparation of isolated antibody obtained from an animal exposed to the vaccine or immunogenic composition of claim 9 wherein the preparation comprises antibodies that bind the isolate of claim 8.

13. An isolated and purified nucleic acid molecule comprising a preselected nucleic acid sequence which encodes a turkey coronavirus S1 polypeptide, a biologically active subunit, or a biologically active variant thereof, the presence of which is associated with spiking mortality of turkeys.

14. The nucleic acid molecule of claim 13 wherein the preselected nucleic acid sequence comprises SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:30.

15. The nucleic acid molecule of claim 13 wherein the preselected nucleic acid sequence encodes a polypeptide having an amino acid sequence comprising SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:31, or a biologically active subunit thereof.

16. The nucleic acid molecule of claim 13 wherein the preselected nucleic acid sequence is operably linked to a promoter functional in a host cell so as to form an expression cassette.

17. The nucleic acid molecule of claim 13 wherein the preselected nucleic acid sequence encodes a fusion polypeptide comprising said S1 polypeptide.

18. An isolated and purified nucleic acid molecule which is complementary to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:30.

19. An isolated and purified turkey coronavirus S1 polypeptide, a biologically active subunit or variant thereof, which is associated with spiking mortality of turkeys.

20. The isolated and purified polypeptide of claim 19 which comprises an amino acid sequence corresponding to SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:31.

21. A fusion polypeptide comprising at least a portion of the isolated and purified polypeptide of claim 19.

22. A vaccine or immunogenic composition comprising an amount of the isolated and purified polypeptide of claim 19 effective to immunize a susceptible turkey against spiking mortality of turkeys.

23. A vaccine or immunogenic composition comprising an amount of the isolated and purified polypeptide of claim 19 effective to immunize a bovine against bovine coronavirus infection.

24. The vaccine of claim 22 or 23 wherein the polypeptide is combined with a physiologically acceptable carrier.

25. A method of using a nucleic acid molecule encoding a turkey coronavirus S1 polypeptide, the presence of which is associated with spiking mortality of turkeys, said method comprising expressing the nucleic acid molecule in a cultured host cell transformed with a chimeric vector comprising said nucleic acid molecule operably linked to control sequences recognized by the host cell.

26. The method of claim 25 wherein the nucleic acid molecule comprises SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:30.

27. The method of claim 25 wherein the polypeptide has an amino acid sequence comprising SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:31.

28. The method of claim 25 which further comprises recovering the polypeptide from the host cell.

29. An expression cassette comprising a preselected DNA segment that is complementary to a) SEQ ID NO:2, b) SEQ ID NO:4 or c) SEQ ID NO:30 which is operably linked to a promoter functional in a host cell.

30. An oligonucleotide which consists of at least about 7-50 nucleotides, and which has at least about 80% identity to, or is complementary to, a nucleotide sequence comprising SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:30.

31. A diagnostic assay for a coronavirus associated with spiking mortality of turkeys, comprising:

(a) contacting an amount of purified turkey coronavirus S1 polypeptide encoded by the genomic nucleic acid of the coronavirus with a physiological sample which comprises antibodies suspected of specifically reacting with the polypeptide, for a sufficient time to form binary complexes between at least a portion of the antibodies and a portion of the purified polypeptide; and

(b) detecting or determining the presence or amount of said binary complexes.

32. A diagnostic assay for a coronavirus associated with spiking mortality of turkeys, comprising:

(a) contacting an amount of isolated, cell culture adapted turkey coronavirus obtained from a turkey having spiking mortality of

turkeys, or obtained from an animal exposed to turkeys having spiking mortality of turkeys, with a physiological sample which comprises antibodies suspected of specifically reacting with the coronavirus, for a sufficient time to form binary complexes between at least a portion of the antibodies and a portion of the isolated coronavirus; and

(b) detecting or determining the presence or amount of said binary complexes.

33. A method for detecting spiking mortality of turkeys, comprising:

(a) contacting an amount of purified turkey coronavirus S1 polypeptide encoded by the genomic nucleic acid of the coronavirus with a physiological sample from a turkey which comprises antibodies suspected of specifically reacting with the polypeptide, for a sufficient time to form binary complexes between at least a portion of the antibodies and a portion of the purified polypeptide; and

(b) detecting or determining the presence of said binary complexes, wherein the presence of said complexes is indicative of a turkey having, or at risk of, spiking mortality of turkeys.

34. A method for detecting spiking mortality of turkeys, comprising:

(a) contacting an amount of isolated, cell culture adapted turkey coronavirus obtained from a turkey having spiking mortality of turkeys, or obtained from an animal exposed to turkeys having spiking mortality of turkeys, with a physiological sample from a turkey which comprises antibodies suspected of specifically reacting with the coronavirus, for a sufficient time to form binary complexes between at least a portion of the antibodies and a portion of the isolated coronavirus; and

(b) detecting or determining the presence of said binary complexes, wherein the presence of said complexes is indicative of a turkey having, or at risk of, spiking mortality of turkeys.

35. The method of claim 31 or 33 wherein the purified polypeptide has an amino acid sequence comprising SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:31.

36. The method of claim 32 or 34 wherein the coronavirus is UGA-APN, UGA-APG, UGA-APBOA or UGA-APBOB.

37. A diagnostic kit for detecting or determining the presence of antibodies that specifically react with a coronavirus which is associated with spiking mortality of turkeys, which comprises packaging, containing, separately packaged:

(a) a solid phase capable of having attached thereto a polypeptide; and

(b) a known amount of a purified coronavirus S1 polypeptide which specifically reacts with antibodies specific for the coronavirus.

38. A diagnostic kit for detecting or determining the presence of antibodies that specifically react with a coronavirus which is associated with spiking mortality of turkeys, which comprises packaging, containing, separately packaged:

(a) a solid phase capable of having attached thereto a virus preparation; and

(b) a known amount of isolated, cell culture adapted coronavirus, the presence of which is associated with spiking mortality of turkeys, that specifically reacts with antibodies specific for the coronavirus.

39. A method to prevent or inhibit spiking mortality of turkeys, comprising: immunizing a turkey with an effective amount of purified coronavirus S1 polypeptide.

40. A method to prevent or inhibit spiking mortality of turkeys, comprising: immunizing a turkey with an effective amount of isolated, cell culture adapted coronavirus, wherein the virus is obtained from a turkey having, or an animal exposed to a turkey having, spiking mortality of turkeys.

41. The method of claim 40 wherein the isolate is killed prior to immunization.

42. The method of claim 40 wherein the isolate is not killed prior to immunization.

43. A turkey immunized with an effective amount of purified coronavirus S1 polypeptide, wherein said immunized turkey produces protective antibodies to a coronavirus associated with spiking mortality of turkeys.

44. A turkey immunized with an effective amount of isolated, cell culture adapted coronavirus obtained from a turkey having, or an animal exposed to turkeys having, spiking mortality of turkeys, wherein said immunized turkey produces protective antibodies to a coronavirus associated with spiking mortality of turkeys.

45. A bovine immunized with an effective amount of purified turkey coronavirus S1 polypeptide, wherein the immunized bovine produces protective antibodies to bovine coronavirus.

46. A bovine immunized with an effective amount of isolated, cell culture adapted turkey coronavirus obtained from a turkey having, or an animal

exposed to turkeys having, spiking mortality of turkeys, wherein the immunized bovine produces protective antibodies to bovine coronavirus.

47. A diagnostic method, comprising:

(a) contacting an amount of turkey coronavirus DNA obtained by reverse transcription of RNA from a physiological sample from a turkey at risk of or afflicted with spiking mortality of turkeys, or an animal exposed to a turkey at risk of or afflicted with spiking mortality of turkeys, with an amount of at least two oligonucleotides under conditions effective to amplify the DNA by a polymerase chain reaction so as to yield an amount of amplified DNA, wherein at least one oligonucleotide is specific for the nucleic acid of a turkey coronavirus which is associated with spiking mortality of turkeys; and

(b) detecting or determining the presence of the amplified DNA, wherein the presence of the amplified DNA is indicative of a turkey at risk of, or afflicted with, spiking mortality of turkeys, or an animal exposed to said turkey.

48. A method for detecting nucleic acid encoding a turkey coronavirus S1 polypeptide associated with spiking mortality of turkeys, comprising:

(a) contacting an amount of turkey coronavirus DNA obtained by reverse transcription of RNA from a physiological sample from a turkey at risk of or afflicted with spiking mortality of turkeys or an animal exposed to said turkey, with an amount of at least two oligonucleotides under conditions effective to amplify the DNA by a polymerase chain reaction so as to yield an amount of amplified DNA, wherein at least one oligonucleotide is specific for the nucleic acid of a turkey coronavirus which is associated with spiking mortality of turkeys;

(b) detecting or determining the presence of the amplified DNA.

49. A diagnostic kit for detecting the presence of a nucleic acid molecule associated with spiking mortality of turkeys in a sample, which comprises packaging containing (a) a known amount of a first oligonucleotide, wherein the first oligonucleotide consists of at least about 7 to about 50 nucleotides, and wherein the oligonucleotide has at least about 80% identity to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:30, and (b) a known amount of a second oligonucleotide, wherein the second oligonucleotide consists of at least about 7 to about 50 nucleotides, and wherein the oligonucleotide has at least about 80% identity to a nucleotide sequence which is complementary to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:30.

Amino Acid	Codon
Phe	UUU, UUC
Ser	UCU, UCC, UCA, UCG, AGU, AGC
Tyr	UAU, UAC
Cys	UGU, UGC
Leu	UUA, UUG, CUU, CUC, CUA, CUG
Trp	UGG
Pro	CCU, CCC, CCA, CCG
His	CAU, CAC
Arg	CGU, CGC, CGA, CGG, AGA, AGG
Gln	CAA, CAG
Ile	AUU, AUC, AUA
Thr	ACU, ACC, ACA, ACG
Asn	AAU, AAC
Lys	AAA, AAG
Met	AUG
Val	GUU, GUC, GUA, GUG
Ala	GCU, GCC, GCA, GCG
Asp	GAU, GAC
Gly	GGU, GGC, GGA, GGG
Glu	GAA, GAG

FIG. 1

FIG. 2

FIG. 3

FIG. 4

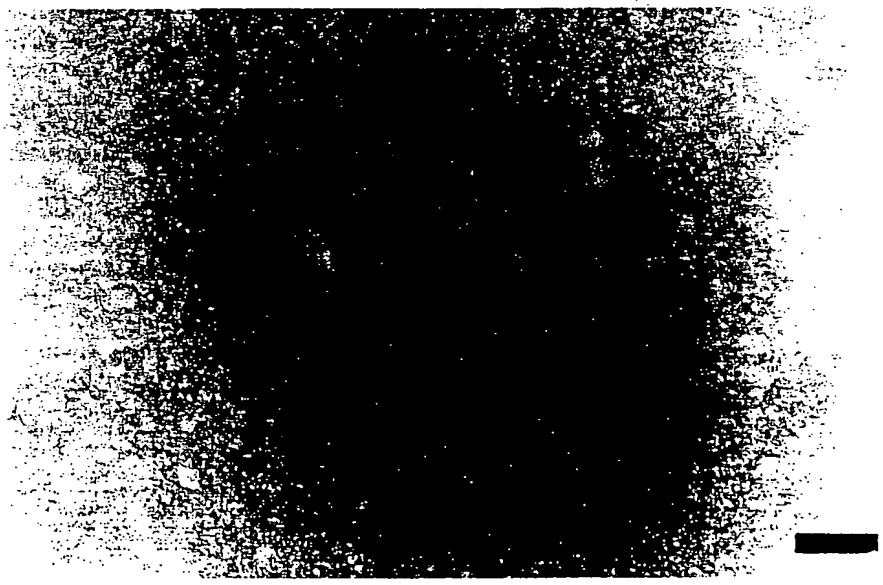


FIG. 5

FIG. 6

FIG. 7

FIG. 8

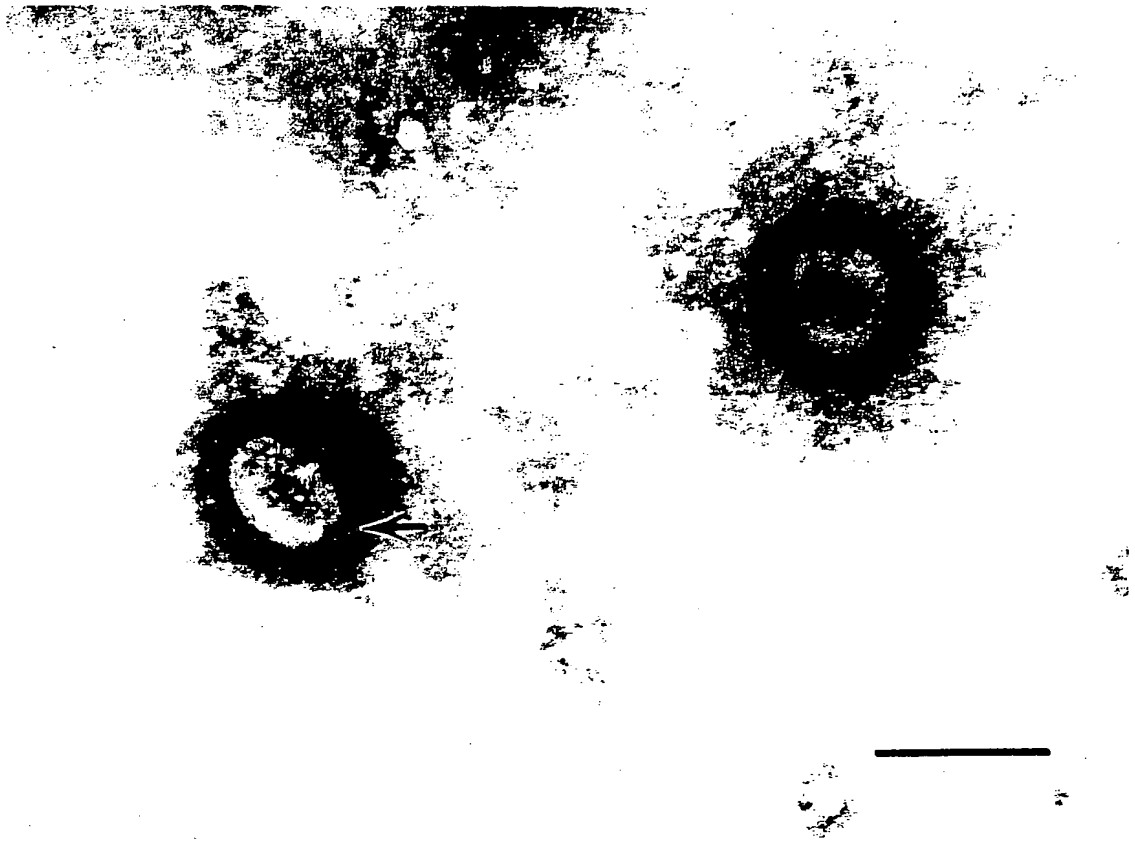


FIG. 9

FIG. 10

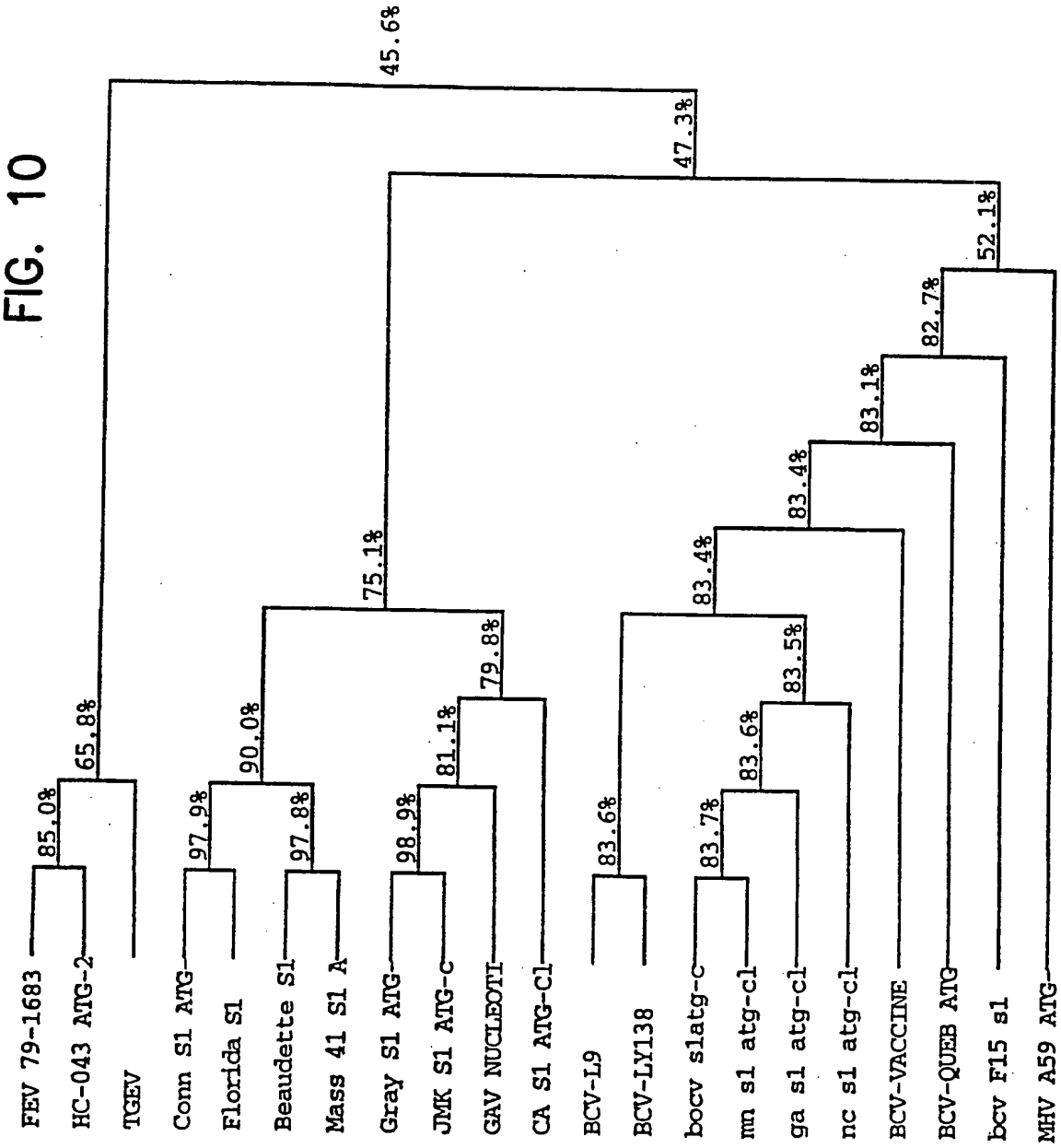
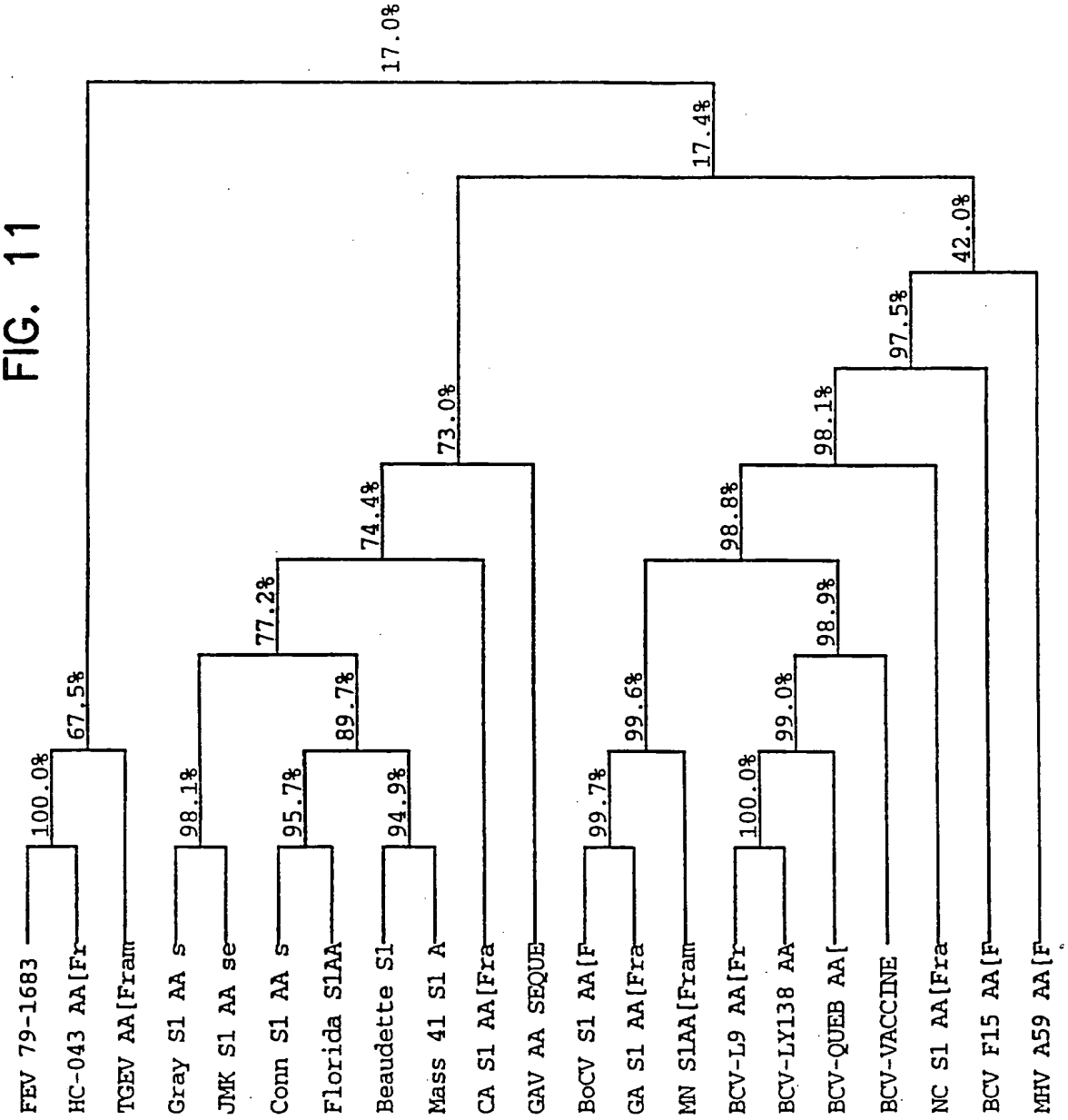


FIG. 11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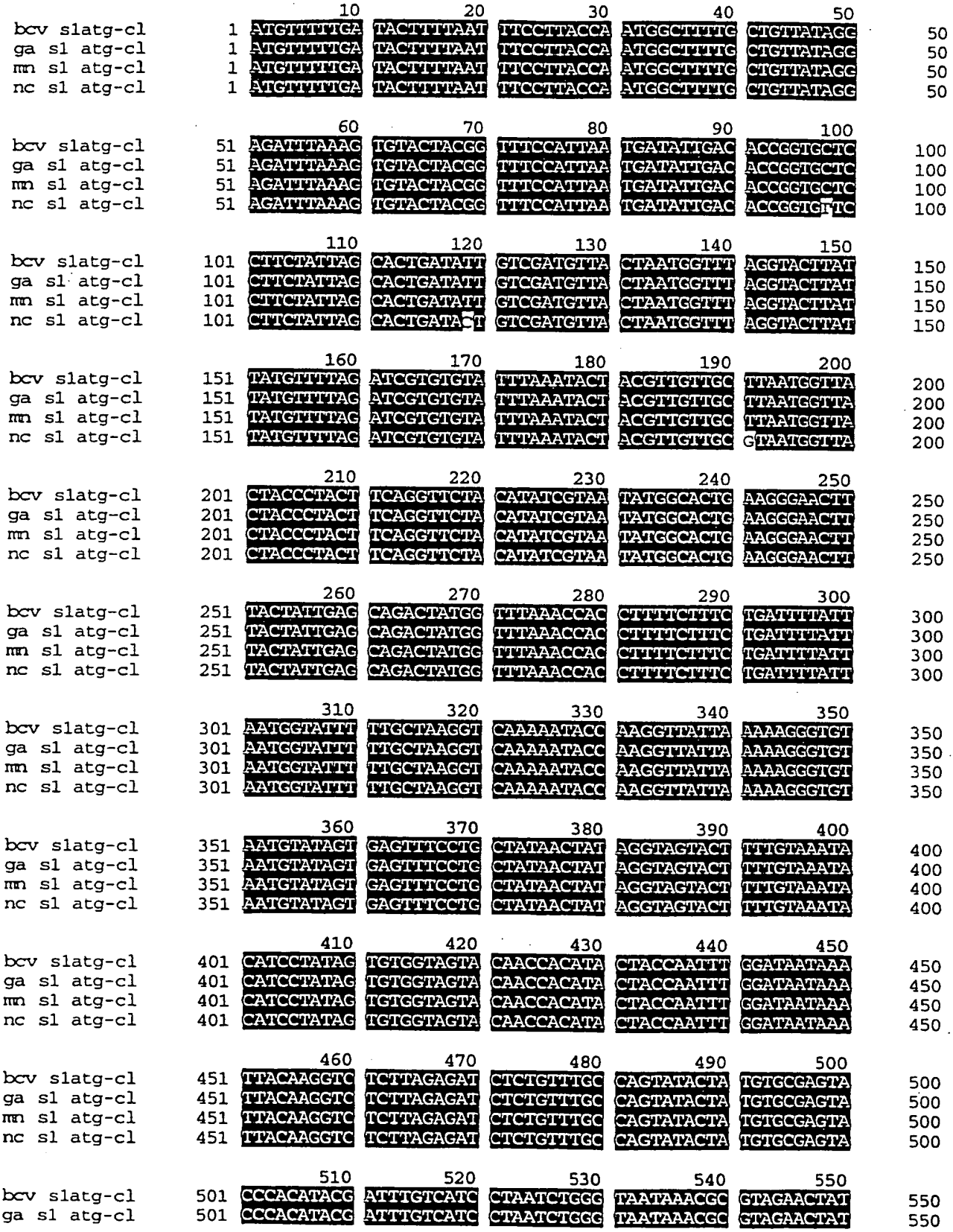

FIG. 12

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                         560        570        580        590        600
bcv s1atg-cl     551 GGCATTGGGA TACAGGTGTT GTTTCCTGTT TATATAAGCG TAATTTCACA  600
ga s1 atg-cl     551 GGCATTGGGA TACAGGTGTT GTTTCCTGTT TATATAAGCG TAATTTCACA  600
mn s1 atg-cl     551 GGCATTGGGA TACAGGTGTT GTTTCCTGTT TATATAAGCG TAATTTCACA  600
nc s1 atg-cl     551 GGCATTGGGA TACAGGTGTT GTTTCCTGTT TATATAAGCG TAATTTCACA  600

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ga s1 atg-cl     601 TATGATGTGA ATGCTGATTA CTTGTATTTC CATTTTTATC AAGAAGGTGG  650
mn s1 atg-cl     601 TATGATGTGA ATGCTGATTA CTTGTATTTC CATTTTTATC AAGAAGGTGG  650
nc s1 atg-cl     601 TATGATGTGA ATGCTGATTA CTTGTATTTC CATTTTTATC AAGAAGGTGG  650

                         660        670        680        690        700
bcv s1atg-cl     651 TACTTTTTAT GCATATTTTA CAGACACTGG TGTTGTGACT AAGTTTCTGT  700
ga s1 atg-cl     651 TACTTTTTAT GCATATTTTA CAGACACTGG TGTTGTGACT AAGTTTCTGT  700
mn s1 atg-cl     651 TACTTTTTAT GCATATTTTA CAGACACTGG TGTTGTGACT AAGTTTCTGT  700
nc s1 atg-cl     651 TACTTTTTAT GCATATTTTA CAGACACTGG TGTTGTGACT AAGTTTCTGT  700

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ga s1 atg-cl     701 TTAATGTTTA TTTAGGCACG GTGCTTTCAC ATTATTATGT CCTGCCTTTG  750
mn s1 atg-cl     701 TTAATGTTTA TTTAGGCACG GTGCTTTCAC ATTATTATGT CCTGCCTTTG  750
nc s1 atg-cl     701 TTAATGTTTA TTTAGGCACG GTGCTTTCAC ATTATTATGT CCTGCCTTTG  750

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ga s1 atg-cl     751 ACTTGTTATA GTGCTATGAC TTTAGAATAT TGGGTTACAC CTCTCACTTC  800
mn s1 atg-cl     751 ACTTGTTATA GTGCTATGAC TTTAGAATAT TGGGTTACAC CTCTCACTTC  800
nc s1 atg-cl     751 ACTTGTTATA GTGCTATGAC TTTAGAATAT TGGGTTACAC CTCTCACTTC  800

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ga s1 atg-cl     801 TAAACAATAT TTACTAGCTT TCAATCAAGA TGGTGTTATT TTTAATGCTG  850
mn s1 atg-cl     801 TAAACAATAT TTACTAGCTT TCAATCAACA TGGTGTTATT TTTAATGCTG  850
nc s1 atg-cl     801 TAAACAATAT TTACTAGCTT TCAATCAAGA TGGTGTTATT TTTAATGCTG  850

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ga s1 atg-cl     851 TTGATTGTAA GAGTGATTTT ATGAGTGAGA TTAAGTGTAA AACACTATCT  900
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ga s1 atg-cl     901 ATAGCACCAT CTACTGGTGT TTATGAATTA AACGGTTACA CTGTTCAGCC  950
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nc s1 atg-cl     901 ATAGCACCAT CTACTGGTGT TTATGAATTA AACGGTTACA CTGTTCAGCC  950

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ga s1 atg-cl     951 AATTGCAGAT GTTTACCGAC GTATACCTAA TCTTCCCGAT TGTAATATAG 1000
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ga s1 atg-cl    1001 AGGCTTGGCT TAATGATAAG TCGGTGCCCT CTCCATTAAA TTGGGAACGT 1050
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nc s1 atg-cl    1001 AGGCTTGGCT TAATGATAAG TCGGTGCCCT CTCCATTAAA TTGGGAACGT 1050
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FIG. 12 CONTINUED

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mn s1 atg-cl      1301  CTGCTGCTAA TGTTTCTGTT AGCAGGTTTA ATCCTTCTAC TTGGAATAGG 1350
nc s1 atg-cl      1301  CTGCTGCTAA TGTTTCTGTT AGCAGGTTTA ATCCTTCTAC TTGGAATAGG 1350

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ga s1 atg-cl      1351  AGATTTGGTT TTACAGAACA ATCTGTTTTT AAGCCTCAAC CTGTAGGTGT 1400
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                        1410       1420       1430       1440       1450
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ga s1 atg-cl      1401  TTTTACTCAT CATGATGTTG TTTATGCACA ACATTGTTTT AAAGCTCCCA 1450
mn s1 atg-cl      1401  TTTTACTCAT CATGATGTTG TTTATGCACA ACATTGTTTT AAAGCTCCCA 1450
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                        1510       1520       1530       1540       1550
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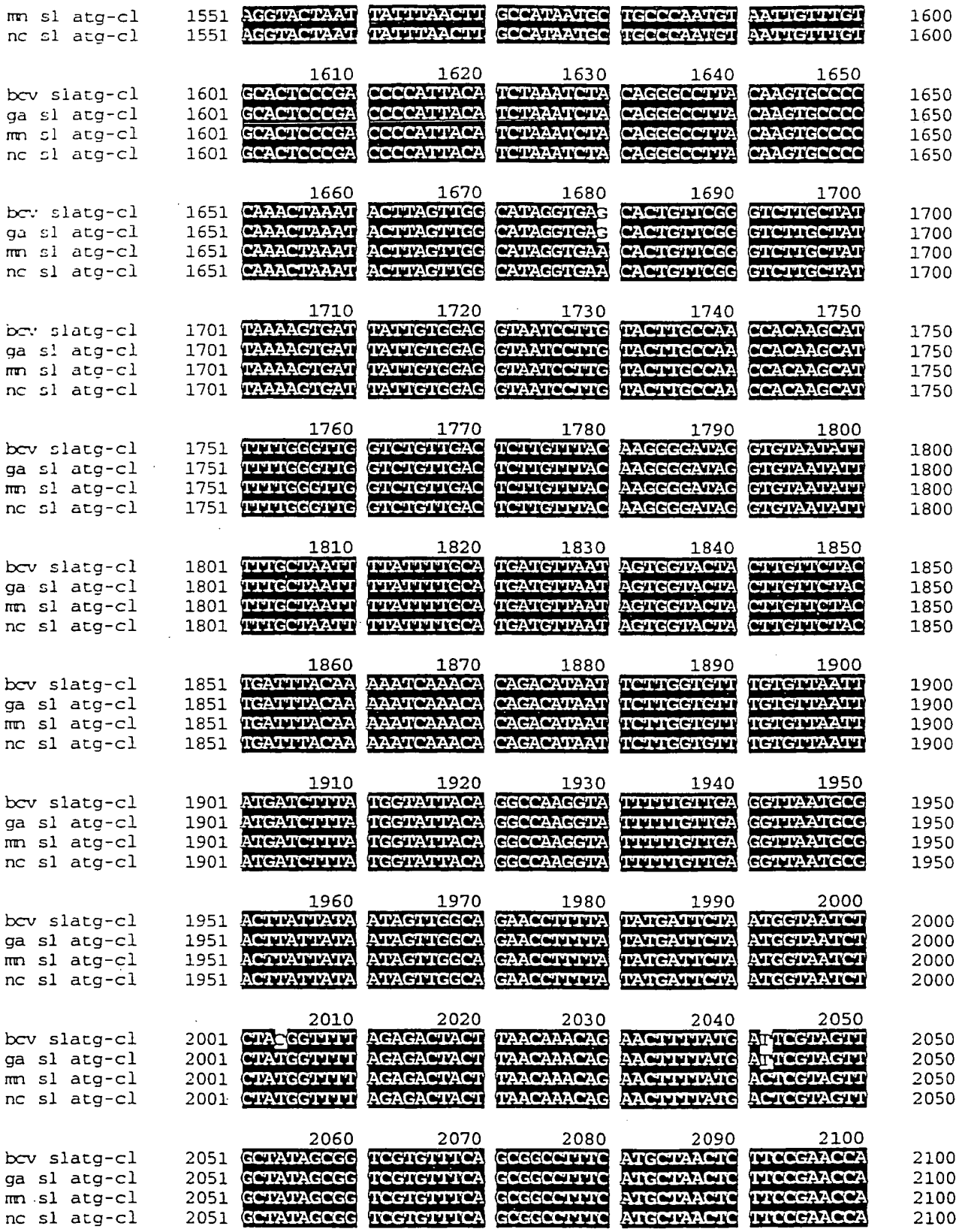

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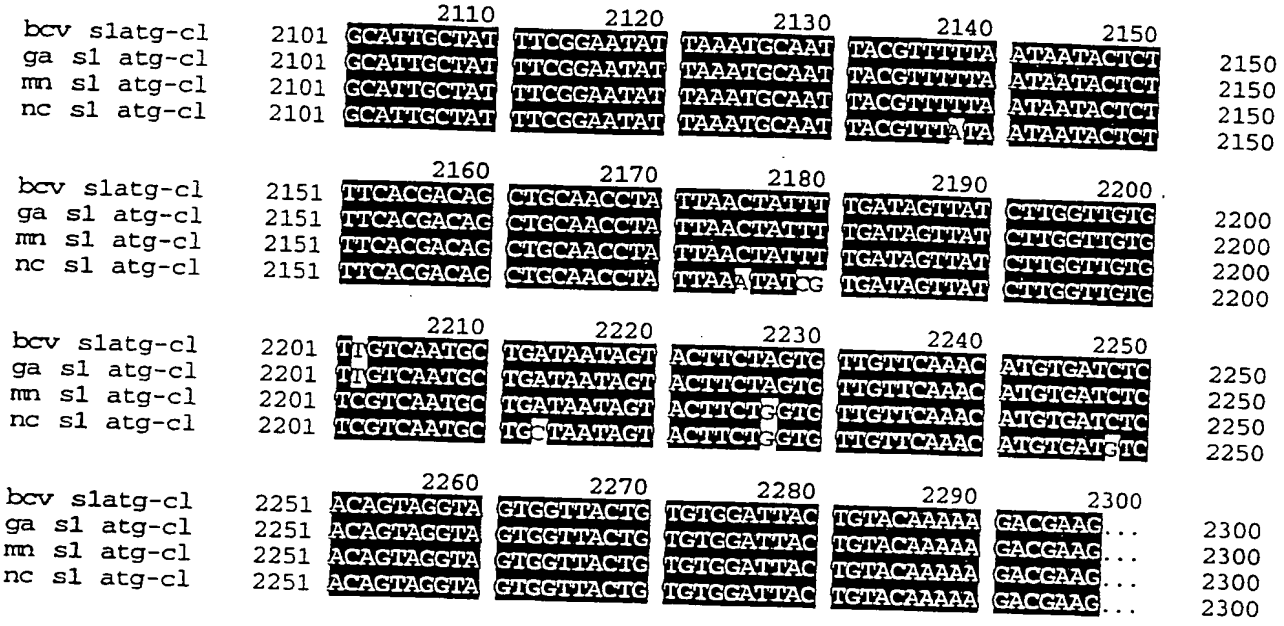

FIG. 12 CONTINUED

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/24313

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/50 C12N7/00 A61K39/215 C07K16/10 C07K14/165
G01N33/569 C12Q1/70 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KÜNKEL F AND HERRLER G: "Structural and functional analysis of the S proteins of two human coronavirus OC43 strains adapted to growth in different cells" ARCHIVES OF VIROLOGY, vol. 141, no. 6, 1996, pages 1123-1131, XP002099167 see the whole document ---	1,2,7, 19,21-30
X	US 5 672 350 A (PARKER M D ET AL.) 30 September 1997 see column 22 - column 24; figure 3; example 1 ---	8-30,45, 46
X	WO 93 23421 A (MILLER T J ET AL.) 25 November 1993 see page 7, line 3 - line 14 ---	19-24
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 April 1999	22/04/1999

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ational Application No
PCT/US 98/24313

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	GUY J S ET AL.: "Antigenic characterization of a Turkey Coronavirus identified in poult enteritis- and mortality syndrome affected turkeys" AVIAN DISEASES, vol. 41, no. 3, July 1997 - September 1997, pages 583-590, XP002099168 see page 585 - page 586 ---	1,2,7
A	BROWN T P ET AL.: "Spiking mortality of turkey poults: 2. Effect of six different in vitro disinfection techniques on organ homogenates capable of reproducing SMT" AVIAN DISEASES, vol. 41, no. 4, October 1997 - December 1997, pages 906-909, XP002099169 ---	39-44
A	WO 95 34686 A (STORZ J) 21 December 1995 see page 4, line 15 - page 6 ---	1-6
X	DEA S ET AL.: "Antigenic and genomic relationships among turkey and bovine enteric coronaviruses" JOURNAL OF VIROLOGY, vol. 64, no. 6, June 1990, pages 3112-3118, XP002099170 AMERICAN SOCIETY FOR MICROBIOLOGY US see the whole document -----	8-12

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 24313

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 39-42 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 98/24313

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		DE 69013512 D	24-11-1994
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		US 5677179 A	14-10-1997